DEPARTAMENT DE MEDICINA

ELUCIDATING THE GENETIC SUSCEPTIBILITY OF HYPERTENSION ASSOCIATED MICROALBUMINURIA: GENOME WIDE SCAN.

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UNIVERSITAT DE VALÈNCIA
Servei de Publicacions
2010

Aquesta Tesi Doctoral va ser presentada a València el dia 23 de març de 2010 davant un tribunal format per:

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Va ser dirigida per:
Dr. Felipe Javier Chaves Martínez
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Dipòsit legal: V-2096-2011
I.S.B.N.: 978-84-370-7809-0
Edita: Universitat de València
Servei de Publicacions
C/ Arts Gràfiques, 13 baix
46010 València
Spain
Telèfon:(0034)963864115

UNIVERSITY OF VALENCIA

ELUCIDATING THE GENETIC SUSCEPTIBILITY OF HYPERTENSION ASSOCIATED MICROALBUMINURIA: GENOME WIDE SCAN

Doctoral Thesis presented by:
Fernando Martínez García,
In order to obtain the grade of
Medical Doctor.
Director of the thesis:
Dr. F. Javier Chaves Martínez and
Professor Josep Redón i Más.

VALENCIA, FEBRUARY, 2010

UNIVERSITY OF VALENCIA

Felipe Javier Chaves Martínez, doctor in biological sciences, degree in biology and specialist in genetics, and Josep Redón Mas, professor of medicine of the University of Valencia.

To Whom It May Concern:

The doctoral thesis**, ELUCIDATING THE GENETIC SUSCEPTIBILITY OF HYPERTENSION ASSOCIATED MICROALBUMINURIA: WHOLE GENOME SCAN,** presenting by Fernando Martínez García and being directed by doctors F. Javier Chaves Martínez and Josep Redón Mas, is an important contribution to the genetics of renal damage in essential hypertension and it is suitable to be presented and defended in front of the corresponding tribunal.

In Valencia, February of 2010.

Signature of the director of the thesis
Dr. F. Javier Chaves Martínez

Signature of the co-director of the thesis
Professor Josep Redón i Más

... knowledge must continually be renewed by ceaseless effort, if it is not to be lost. It resembles a statue of marble which stands in the desert and is continually threatened with burial by the shifting sand. The hands of service must ever be at work, in order that the marble continue to lastingly shine in the sun. To these serving hands mine shall also belong.

Albert Einstein, 1950

Acknowledgements

This doctoral thesis has been done within the program “Contratos post-formación sanitaria especializada” of the National Health Institute Carlos III.

I would like to acknowledge, first and foremost, to the doctors, Josep Redon and Javier Chaves who have trusted on me to carry out this project.
Secondly, I want to acknowledge to those who helped me during my stay in the Glasgow Cardiovascular Research Centre, in special: Dra. Chiara Taurino, Dra. María Moreno, Dra. Samantha Madrazo, Dr Raul Alba and Dr. Ulf Nesius for their support; Dr Sandosh Padmanabhan for introduce me in the genetic analysis and in the wonderful world of call rates, Q-Q plots and cluster plots; Professor Anna Dominiczak, director of the GCRC, and Dr Christian Delles for organize my stay in that important research centre; To Margaret Kinninmont for being so kind with me and Jillian Blair for help me to find a place where to live in Glasgow.
To our colleagues Dr Jose María Pascual and Dr Francisco Perez of Sagunto`s hospital who were in charge for the recruitment of patients in the hospital of Sagunto and helped me with the clinical database.
To the Professor Richard Cooper who as a director of the epidemiology department of the Loyola University in Chicago, helped me to interpret the results and put me in contact with Dr Bamidele Tayo who has been very important in the downstream analysis; To Dr Ramon Durazo for help me to understand the complex world of the statistic.
To the people of the microarray lab within the central research unit of the Medicine Faculty (University of Valencia) who performed the genotyping procedure, especially to Eva Serna who solved some of the technical questions related with the genotyping procedure.
To Dra.María Luisa Mansego who has been my support in the genetic lab during three years and has helped me to organize the information.
To all the members of the Internal Medicine department, especially those who have heard my complaints during those long hours that I spent in the office called "Estar de Malats". To all the people who are involved in clinical research within the Hypertension Unit, in special: Elena, Oscar, Miriam and Antonio Vicente for that shorts breaks.
To my family and all of my friends who have tried to make these busy months more easygoing.

Acronyms (arrange by alphabetical order)

ABPM,	Ambulatory blood pressure monitoring.
ACE	Angiotensin I converting enzyme.
ACEi,	Angiotensin I converting enzyme inhibitors.
ACR,	Albumin creatinine ratio.
ADAM23,	A disintegrin and metalloprotease domain 23.
ADAM7,	Disintegrin and metalloproteinase domain-containing protein 7 precursor gene.
ADD1,	Alpha adducin gene.
ADIPOQ,	Adiponectin precursor.
ADLR1,	Aldose reductase gene.
ADRBR2,	Beta adrenergic receptor type 2 gene.
AGE,	Advanced glycation end products.
AGT,	Angiotensinogen.
ANKAR,	Ankyrin and armadillo repeat-containing protein gene.
ANP,	Atrial natriuretic peptide.
Apo E,	Apolipoprotein E.
apt,	Affymetrix power tool.
AQP1	Aquaporin 1 gene.
ARBs,	Angiotensin II receptor blockers.
ARBs,	Angiotensin II receptor blockers.
ASNSD1,	Asparagine synthetase domain-containing protein 1 gene.
AT1R, AGTR1	Angiotensin II type 1 receptor gene.
AUC,	Area under curve.
Bcl-2,	B-cell leukaemia/lymphoma 2 gene.
BMI,	Body mass index.
BNC2,	Basonuclin gene.
BONF,	Bonferroni correction test..
BP,	Blood pressure.
BRLMM,	Bayesian robust linear model with Mahalanobis distance classifier.
BSA,	Body surface area.
C12orf59,	Chromosome 12 open reading frame 59 gene.
CC,	Creatinine clearance.
CDH8,	Cadherin-8 precursor.
CDH5,	Cadherin-5 precursor.

CDH11,	Cadherin 11 precursor gene.
CDKN2A,	Cyclin-dependent kinase inhibitor 2A.
CDKN2B,	Cyclin-dependent kinase inhibitor 2B
CETP,	Cholesterol ester transfer protein precursor.
CG,	Candidate genes.
Chr,	Chromosome.
CKD,	Chronic kidney disease
CNDP1,	Carnosine dipeptidase 1 gene.
CNV,	Copy number variation.
COL4A1	Collagen type 4 Alpha 1 gene.
CRF,	Chronic renal failure.
CRP,	C reactive protein.
CV,	Cardiovascular.
CXCL12,	Chemokine (C-X-C motif) ligand 12 gene.
CYP11B2,	Aldosterone synthase gene.
DBP,	Diastolic blood pressure.
DF,	Degree freedom.
DM,	Dynamic model.
DM2,	Type 2 diabetes mellitus.
DN,	Diabetic nephropathy.
DNA,	Deoxyribonucleic acid.
DSP,	Discordant sib-pairs.
EDTA,	Ethylenediaminetetraacetic acid.
eGFR,	Estimated GFR.
ELMO1,	Engulfment and cell motility 1 gene.
ELMO3,	Engulfment and cell motility protein 3.
EM,	Expectation maximization algorithm.
eNOS,	Endothelial nitric oxide synthase.
ENPP1,	Ectonucleotide pyrophosphatase phosphodiesterase 1 gene.
EPDR1,	Ependymin related protein 1 gene.
EPHA5,	Ephrin-A5 precursor gene.
ESRD,	End stage renal disease.
FBAT,	Family based association test.
FDR,	False discovery rate.
FSGS,	Focal and segmental glomerulosclerosis.

GCOS,	GeneChip Operating Software.
GEE ,	Generalized estimating equations.
GFR,	Glomerular filtrate rate.
GLEPP1,	Glomerular epithelial protein 1.
GLM,	Generalized linear model.
GRR,	Genotypes relative risk.
GTYPE,	GeneChip Genotyping Analysis Software.
GWAS,	Genome wide association studies.
HDL,	High density lipoprotein.
hNPY,	Human neuropeptide Y.
HOMA,	Homeostatic model assessment index.
HTN,	Essential hypertension.
HWE,	Hardy-Weinberg equilibrium.
I/D,	Insertion-Deletion polymorphism.
IBD,	Identity by descent.
IBS,	Identity by state.
IFNA,	Interferon alpha precursor.
IFNB1,	Interferon beta precursor.
IFNW1,	Interferon omega-1 precursor.
IFNγ,	Interferon gamma
IGF1R,	Insulin growth factor 1 receptor.
IGF2BP2,	Insulin-like growth factor 2 mRNA-binding protein 2.
IL-1,	Interleukin 1
IL-6,	Interleukin 6.
IL-8,	Interleukin 8.
IL-33,	Interleukin 33.
IMPAD1,	Inositol monophosphatase domain containing 1 gene.
IMT,	Intima media thickness.
IP-10,	Interferon-gamma-inducible protein.
KLRC2,	NKG2-C type II integral membrane protein gene.
KLRC3,	NKG2-E type II integral membrane protein gene.
KLRC4,	NKG2-F type II integral membrane protein gene.
KLRD1,	Natural killer cells antigen CD94 gene.
KLRK1,	Killer cell lectin-like receptor subfamily K member 1 gene.
KSR2,	Kinase suppressor of Ras 2.
LA,	Linkage analysis.

LD,	Linkage disequilibrium.
LDL,	Low density lipoprotein.
LOD,	Logarithmic of odds score.
LOX1,	Oxidized low-density lipoprotein receptor 1.
LPL,	Lipoprotein lipase.
LR,	Logistic regression.
LVM,	Left ventricular mass.
MAF,	Minor allele frequency.
MALD,	Mapping by admixture linkage disequilibrium.
MCF2L2,	MCF2 cell line derived transforming sequence-like 2 gene.
MCTP2,	Multiple C2 and transmembrane domain-containing protein 2 gene.
MCP-1,	Monocyte chemoattractant protein-1.
MDRD,	Modification of diet in renal disease.
MIP-1δ,	Macrophage inflammatory protein-1delta.
MLS	Maximum likelihood score.
MnSOD,	Manganese superoxide dismutase.
MSR1,	Macrophage acetylated LDL receptor I and II gene.
MTHFS,	Methenyltetrahydrofolate synthetase.
MYH9,	Non-muscle myosin heavy chain 9 gene.
NADPH,	Nicotiamide-adenin dinucleotid phosphate.
NCALD,	Neurocalcin D gene.
NRP1,	Neuropilin 1 gene.
OGGT	Oral glucose tolerance test.
OLR1,	Oxidized low-density lipoprotein receptor 1 gene.
OR,	Odds ratio.
ORMDL1,	ORM1-like1.
OSA,	Ordered subsets analysis.
OSGLPL1,	O-sialoglycoprotein endopeptidase-like 1 gene.
PAI-I1	Plasminogen activator inhibitor type 1.
PCDH9,	Protocadherin 9.
PCDH10,	Protocadherin-10 precursor.
PCDH18,	Protocadherin-18 precursor.
PK-C,	Protein kinase C.
PLEKHH2,	Pleckstrin homology domain containing, family H gene.
PON-1,	Paraoxonase 1.
PON-2,	Paraoxonase 2.

PTPRO,	Protein tyrosine phosphatase receptor type O.
PVT1,	Plasmocytoma variant translocation gene.
QC,	Quality control
Q-Q,	Quantile-Quantile.
QTL,	Quantitative trait loci.
RAAS,	Renin angiotensin aldosterone system.
RAGE,	Receptor of the advanced glycation end products.
REEP1,	Receptor Expression-Enhancing Protein 1.
RFC5,	Replication factor C subunit 5.
RFLP,	Restriction fragment length polymorphism.
RI,	Renal insufficiency.
SBP,	Systolic blood pressure.
SD,	Standard deviation
SFRP4,	Secreted frizzled-related protein 4 precursor gene.
SLC12A3,	Solute carrier family 12, member 3 gene.
SLC40A1,	Solute carrier family 40 member 1.
SLE,	Systemic lupus erythemathosus.
SNP,	Single nucleotide polymorphisms.
SNS,	Sympathetic nervous system.
TAOK3,	Serine/threonine-protein kinase TAO3.
TDT,	Transmission disequilibrium test.
TGF-B,	Transforming growth factor beta.
TGFBR1,	TGF-B receptor type 1 gene
TGFBR2,	TGF-B receptor type 2 gene.
TNF-α	Tumor necrosis factor alpha.
TRPC1,	Transient receptor potential cation channel subfamily C, member 1 gene.
TXNDC3,	Thioredoxin domain-containing protein 3 gene.
U6,	U6 spliceosomal RNA gene.
U8,	U8 small nucleolar RNA gene.
UAE,	Urinary albumin excretion.
UTE,	Urinary transferrin excretion.
VEGF,	Vascular endothelial growth factor.
WSB2,	WD repeat and SOCS box-containing protein 2.
WWOX,	WW domain containing oxidoreductase gene.

Genetic glossary

Additive genetic model - In a disease association study, if the risk conferred by an allele is increased r -fold for heterozygotes and 2r -fold for homozygotes, this corresponds to additive model. These data are best analyzed using Armitage trend test for genotype frequencies or by logistic regression in which the genotypes are represented as (-1), 0, (+1). This genotype -based association test does not require the locus to be in Hardy -Weinberg equilibrium. In the case of an association with heterozygosity, the additive model test may be statistically non -significant despite the presence of an association. Thus, a non -significant additive model test does not rule out an association. It has been pointed out that genes do not generally act in a simple additive manner but through complex networks involving gene -gene and gene - environment interactions

Admixture mapping (mapping by admixture linkage disequilibrium -MALD) - An association -based approach to localizing disease -causing variants that differ in frequency between two historically separated populations by a whole - genome scan. Fundamental to the use of admixture mapping is the knowledge that the disease of interest exhibits frequency differences across the two populations because of genetic differences.

Affected sibpair (ASP) method - A linkage study design that tests for excess sharing of marker alleles identical by descent in affected -affected sibpairs. This method is often described as a nonparametric and model -free alternative to the parametric LOD score method.

Allele - If the DNA sequence at a given locus (often a gene or a marker) varies between different chromosomes in the population, each different version is an allele. If there are two alleles at a given locus, the allele that is less common in the population is the minor allele.

Association - Comparison of the chance that people who have a particular genetic variation in their DNA have a particular characteristic (trait), symptom, or disease with the chance that people who do not have the particular genetic variation have the particular characteristic, symptom, or disease.

Candidate gene - . A gene known to be located in the region of interest whose product has biochemical or other properties suggesting that it may prove to be the disease gene being sought.

Candidate gene approach - This approach involves assessing the association between a particular allele (or set of alleles) of a gene that may be involved in the disease (i . e . , a candidate gene) and the disease itself. In other words, this type of association study tries to answer the question, “ Is one allele of a candidate gene more frequently seen in subjects with the disease than in subjects without the disease?”.

Case -control study: A design preferred over cohort studies for relatively rare diseases in which cases with a disease or exposure are compared with controls randomly selected from the same study base. This design yields odds ratio (as opposed to relative risk from cohort studies) as the measure of the strength of association.

Centimorgan - 1 centimorgan (cM) corresponds to a region within which a crossover is expected once every 100 meioses. This implies a 1% chance of a single crossover at a single meiosis, and because the probability of a double crossover is exceedingly small (about 0·01%), this also corresponds to a chance of roughly 1% of recombination at each meiosis.

Chromosome - One of the threadlike "packages" of genes and other DNA in the nucleus of a cell. Humans have 23 pairs of chromosomes, 46 in all: 44 autosomes (or non -sex chromosomes) and two sex chromosomes. Each parent contributes one chromosome to each pair, so children get half of their chromosomes from their mothers and half from their fathers.

Cohort study: A longitudinal follow -up study which begins with a group of people who do not have the trait of interest at the outset but a proportion of which will develop during the follow -up. The outcome is modeled for the explanatory variables to obtain the relative risk. Cohort studies may be historical or prospective.

Complex disease - The term complex trait/disease refers to any phenotype that does not exhibit classic Mendelian inheritance attributable to a single gene; although they may exhibit familial tendencies (familial clustering, concordance among relatives). The contrast between Mendelian diseases and complex diseases involves more than just a clear or unclear mode of inheritance. Other hallmarks of complex diseases include known or suspected environmental risk factors; seasonal, birth order, and cohort effects; late or variable age of onset; and variable disease progression.

Dominant allele - An allele that masks an alternative allele when both are present (in heterozygous form). Homozygous dominant and heterozygous genotypes contribute the same to the phenotype. Most common autosomal dominant diseases are due to mutations in transcription factor genes

Dominant model - A genetic association analysis mode that examines association with a dominant allele. The comparison groups are wild -type homozygous genotypes vs allele positivity (combining heterozygotes and homozygotes for the variant).

EM algorithm - A method for calculating maximum likelihood estimates with incomplete data. E (expectation) -step computes the expected values for missing data and M (maximization) -step computes the maximum likelihood estimates assuming complete data. It was first used in genetics (Ceppellini R et al, 1955) to estimate allele frequency for

phenotype data when genotypes are not fully observable (this requires the assumption of HWE and calculation of expected genotypes from phenotype frequencies)

Exon - A segment of a gene that is represented in the mature RNA product. Individual exons typically include protein -coding sequences.

Gene - the functional and physical unit of heredity passed from parent to offspring. Genes are pieces of DNA, and many genes contain the information for making a specific protein.

Gene family - A group of genes having similar DNA sequence evolved from a single ancestral gene. These genes may or may not be located in the same region of a chromosome.

Genetic marker - a segment of DNA with a known physical location on a chromosome and a discernible inheritance pattern. A marker can be a gene, or it can be a section of DNA with no known function. DNA segments that lie near each other on a chromosome tend to be inherited together. Therefore, markers often are used as indirect ways of tracking the inheritance pattern of a gene that has not yet been identified, but whose approximate location is known.

Genetic variation (variant) - differences (or variants) in DNA sequences that are found by comparing the genomes of different individuals and can be used as genetic markers to track inheritance patterns in families.

Genome - all the DNA contained in an organism or a cell, which includes the chromosomes within the nucleus and the DNA in organelles called mitochondria.

Genomic control - One method to adjust for population stratification bias in case -control association studies is to use a 'genomic control markers' panel. The panel consists of 20 -50 polymorphic markers unlinked to the loci of interest. The information obtained from unlinked markers may be used in a variety of ways (genomic control, structured association, latent -class approach). The adjustments requires some statistical manipulation, which can be handled using a variety of statistical approaches.

Genotype - all or part of the genetic make -up of an individual or group, including variation at a particular genetic marker or gene.

Genotype -environment (GxE) interaction - This term refers to both the modification of genetic risk factors by environmental risk and protective factors, and the role of specific genetic risk factors in determining individual differences in vulnerability to environmental risk factors. When GxE interaction is present, a specific environmental change

influences the outcome in different ways depending on the genotype. This requires inclusion of a multiplicative interaction term into the statistical model.

Genotype relative risk (GRR) - The risk of disease for one genotype at a locus versus another. It is usually assessed as having one copy of the allele of interest (Aa) vs having none (AA), which is GRR1; and having two copies of the allele (aa) vs having none, which is GRR2. In simple statistical analysis this is achieved by using dummy variables for each genotype, selecting the genotype AA as referent and obtaining odds ratios for other genotypes Aa and aa. Most of the time, what is presented is actually genotype odds ratio.

Genotyping - the process whereby the genotype(s) of an individual or many individuals is (are) determined from a DNA sample(s) in the laboratory. Typically, DNA samples are obtained by drawing a small amount of blood or by collecting cheek cells.

GWAS (Genome -wide Association Studies) - research studies that involve scanning markers (genotypes) across the complete set of DNA, or genomes, of many people to find genetic variations associated with a particular disease.

Hardy -Weinberg equilibrium (HWE) - In an infinitely large population, gene and genotype frequencies remain stable as long as there is no selection, mutation, or migration. For a bi -allelic locus where the gene frequencies are p and q: $p^2+2pq+q^2 = 1$. HWE should be assessed in controls in a case -control study and any deviation from HWE should alert for genotyping errors (Gomes, 1999; Lewis, 2002). Relying only on HWE tests to detect genotyping errors is not recommended as this is a low power test (Leal, 2005).

Haplotype - A series of alleles at linked loci along a single chromosome.

Haplotype blocks - A chromosomal region with high linkage disequilibrium and low haplotype diversity. Probably flanked by recombinational hotspots, haplotype blocks are shorter in African populations (average 11kb) than in other populations (average 22kb) (Gabriel, 2002). Haplotype block lengths correlate with recombinational rate (Greenwood, 2004) but most haplotype -block boundaries do not occur at hotspots (Wall, 2003). All pairs of polymorphisms within a block are expected to show high linkage disequilibrium. Haplotype blocks are useful in association studies and a representative set of haplotype tagging SNPs can be used instead of the whole set of polymorphisms within a block (Zhang, 2004). Haploview is the most popular software for haplotype block analysis.

Haplotype relative risk (HRR) method - This method uses non -inherited parental haplotypes of affected persons as the control group and thus eliminates the potential problems of using unrelated individuals as controls in case -control association studies. Haplotyping is not necessary to use this method; it can be used for allelic associations.

Heritability - Fraction of the total phenotypic variation in a population that is caused by genetic differences between individuals: *genetic variance / total variance.* The *genetic variance* is the part of the total variance that is caused by allelic variations at whatever loci influence the trait. The *total variance* is the amount of variation in *phenotype* in a defined population. It only applies to a population on which observations are made and cannot be extended to other populations that have different allele frequencies or environments. Therefore, it cannot be used to explain differences between populations.

High -throughput genotyping - Simultaneous genotyping of large numbers of samples. Most machines can run 4x96 (384) samples simultaneously (SNP typing, real -time PCR, sequencing) with a queuing system that would allow automatic continuation of the typing. A number of companies perform SNP high -throughput genotyping

Identity by descent (IBD) - Alleles that trace back to a shared ancestor. For sibs, refers to inheritance of the same allele from a given parent.

Identical -by -state (IBS) - Two alleles (from the same or different individuals) are identical -by -state if they are of the same type.

Intron - Non -coding DNA that separates neighboring exons in a gene.

Linkage analysis - Strategy for *gene mapping* by testing for linkage between *markers* and *phenotypes* using families. In classic linkage analysis the transmission model is fixed (possibly with parameter values obtained from segregation analysis) and the likelihoods (*LOD scores*) of the disease and marker data are compared under the null hypothesis of no linkage and the alternative hypothesis of linkage. Non -parametric linkage analysis avoids fixing an explicit mode of inheritance of the disease.

Linkage disequilibrium - A condition in which alleles at two *loci* or *genes* are found together in a population at a greater frequency than that predicted simply by the product of their individual allele frequencies. Alleles at markers near disease causing genes tend to be in linkage disequilibrium in the affected individuals. This is particularly the case in isolated, homogeneous populations, in which it can be assumed that most affected individuals carry the same mutation.

Locus - A locus is a unique chromosomal location defining the position of an individual gene or DNA sequence. In genetic linkage studies, the term can also refer to a region involving one or more genes, perhaps including noncoding parts of the DNA.

LOD score - A statistical estimate, obtained in *linkage analysis*, which indicates whether alleles at two loci are inherited together more often than expected and are thus likely to be placed near each other on a chromosome. A LOD score is

the ratio of two probabilities: (1) the probability of the observed inheritance of a trait (usually a disease) and alleles at a marker in a pedigree if they were linked given a inheritance model for the trait and a recombination probability between marker and disease, and (2) the probability of the observed inheritance of a trait and marker in a pedigree under the assumption that they are not linked. A LOD score is the logarithm of the ratio of those two probabilities. LOD scores can be added across pedigrees, and are usually taken to indicate significant linkage if they are above three. The recombination fraction that gives the highest LOD score from a marker of known genomic location can be used to map a gene.

Microarray - A novel method of studying large numbers of *genes* simultaneously by automating and miniaturising a hybridisation detection system. The method uses a robot to precisely apply tiny droplets containing *DNA* to glass slides. The labelled probes are allowed to bind to complementary DNA strands on the slides. The slides are put into a scanning microscope that can measure the brightness of each fluorescente dot; brightness reveals how much of a specific DNA fragment is present.

Microsatellite - Microsatellites consist of multiple repeats of a short sequence (typically 2–8 bp) such as: CACACA . . . . The alleles of a microsatellite are differentiated by the number of repeats they involve (eg, CA12 would denote 12 CA repeats in a row).

Misclassification - Errors in the classification of individuals by phenotype, exposures or genotype that can lead to errors in results. The probability of misclassification can be the same across all groups in a study (nondifferential) or vary among groups (differential).

mRNA (messenger RNA) - RNA transcribed from genes undergoes posttranscriptional processing and the resultant mature mRNA is used as the template for the translation process that results in synthesis of a protein.

Multivariable analysis - As opposed to univariable analysis, statistical analysis performed in the presence of more than one explanatory variable to determine the relative contributions of each is (or should be) called multivariable analysis (in practice, however, it is called univariate and multivariate analysis more frequently). It is a method to simultaneously assess contributions of multiple variables or adjust for the effects of known confounders.

Permutation Test - A statistical approach to examine statistical significance of associations based on Monte Carlo methods that accounts for multiple comparisons issue

Phase - Denotes the haplotypic configuration of linked loci. The diplotype U1U3–V1V2 is consistent with two possible phases: (1) U1–V1 on one chromosome and on one chromosome and U3–V1 on the other. If a child receives U1–V1 on

a paternally derived chromosome from a father with diplotype U1U3–V1V2 it either implies that the father was in phase (1) and no recombination has occurred, or he was in phase (2) and there has been recombination.

Phenotype - the observable traits or characteristics of an individual such as hair color, weight, or the presence or absence of a disease. Phenotypic traits are not necessarily genetic.

Polymerase Chain Reaction (PCR) - A procedure for obtaining a large number of copies of a particular segment of *DNA*. The principle depends on the requirement by DNA polymerase of a primer with a 3' end to which nucleotides can be added. Two such synthetic primers define a segment that is replicated in a thermal cycle of denaturation, reannealing (reformation of complementary primer - DNA structure), and replication. Each cycle, which takes two to three minutes, doubles the amount of DNA between the primer boundaries. Thirty cycles would yield 230 copies. PCR has made it possible to characterize extremely small amounts of DNA.

Polymorphism - Implies genetic variation at a designated locus. A locus that is polymorphic has at least two alternative alleles. Unfortunately, polymorphism has alternative, more specific definitions (none universally accepted), an important example being "the existence of two or more genetic variants (alleles, other sequence variants, chromosomal structure variants) at significant frequencies in the population.

Population stratification - An example of 'confounding by ethnicity' in which the co -existence of different disease rates and allele frequencies within population sub -sections leads to a spurious association at the population level. Differing allele frequencies in ethnically different strata in a single population may lead to a spurious association or 'mask' an association by artificially modifying allele frequencies in cases and controls when there is no real association (for this to happen, the subpopulations should differ not only in allele frequencies but also in baseline risk to the disease being studied) (Mark, 1996; Altshuler, 1998). Confounding, cryptic relatedness (which increases overdispersion of the test statistics and leads to inflation of significance levels overall) and selection bias are potential consequences of population stratification (Thomas, 2005). It is notable that the consequences of population structure on association outcomes increases with sample size, i.e., larger sample size is not a remedy for this issue and may make it worse (Marchini, 2004). Case -control association studies can still be conducted by using genomic controls (Devlin, 1999; Pritchard, 1999) even when population stratification is present.

Publication bias - Editors and authors tend to publish articles containing positive findings as opposed to negative result papers. This results in a belief that there is a consistent association while this may not be the case. Plots of relative risks by study may be used to check publication bias in meta -analyses. If publication bias is operating, one would expect that, of published studies, the larger ones report the smaller effects, as small positive trials are more likely to be published than negative ones.

Quantile -Quantile plot (Q -Q plot) - In a GWAS, the Q -Q plot is used to assess the number and magnitude of observed associations compared with the expectations under no association. The nature of deviations from the identity line provide clues whether the observed associations are true associations or may be due to population stratification or cryptic relatedness or something else.

Quantitative character - A character displaying a 'continuous' phenotypic range rather than discrete classes; characters measured rather than counted such as metabolic activity, height, length, width, arm span, body fat content, growth rate, milk production, blood pressure. The genetic variation underlying a continuous character distribution may be the result of segregation at a single genetic locus or more frequently, at numerous interacting loci which produce a cumulative effect on the phenotype (with contributions from the environment). A gene affecting a quantitative character is a quantitative trait locus, or QTL (should be seen as a continuous trait locus).

R project for statistical computing - R is a language and environment for statistical computing and graphics which can be seen as a different implementation of the S language. R and a comprehensive set of programs written for a variety of statistical analysis are all available as Free Software.

Recessive - A trait that is not expressed in heterozygotes (i.e., that can only be expressed in the homozygotes). Most common recessive disease genes are those encoding metabolic enzymes.

Recessive model - A genetic association analysis mode that examines association with a recessive allele. The comparison groups are variant homozygous genotypes vs the rest (combining heterozygotes for the variant and homozygotes for the wild -type allele).

Restriction Fragment Length Polymorphism (RFLP) - Genetic variation at the site where a restriction enzyme cuts a piece of DNA. Such variations affect the ability of the restriction enzyme to cut, and therefore, produce different fragment sizes. Most RFLPs are single base pair changes in the 4–6 bp target sequence of the restriction enzyme. Vice versa, many single nucleotide polymorphisms (SNPs) are RFLPs and can be detected with this technique.

Single nucleotide polymorphism (SNP) - A DNA variant that represents variation in a single base. A common SNP can be defined as a locus at which two SNP alleles are present, both at a frequency of 1% or more. Across the human genome there could be 10 million common SNPs.

Susceptibility gene - A gene that is neither necessary nor sufficient to cause a disease but increases the risk of its development. These low -penetrance genes would be detected by association studies but would show no evidence for linkage with the disease.

Tag SNP - It is a representative single nucleotide polymorphism (SNP) in a region of the genome with high linkage disequilibrium (the non -random association of alleles at two or more loci). It is possible to identify genetic variation without genotyping every SNP in a chromosomal region. Tag SNPs are useful in whole -genome SNP association studies in which hundreds of thousands of SNPs across the entire genome are genotyped.

Trait - Some characteristic of, for instance, a human, that may or may not have a genetic component.

Transmission disequilibrium test (TDT) - A family -based study to compare the proportion of alleles transmitted (or inherited) from a heterozygous parent to a disease -affected child. Any significant deviation from 0.50 in transmission ratio implies an association.

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Introduction

1. BACKGROUND

Cardiovascular diseases and essential hypertension are the leading causes of mortality and morbidity in developed countries including Spain (http://www.isciii.es/htdocs/centros/epidemiologia/epi_enfcardiovasculares.jsp).

Lifestyle modifications continue being the main strategy in order to prevent them. However it is important to know more about the complex physiopathology of this kind of diseases for both, the development of new effective drugs and the identification of those subjects prone to manifest the disease or related traits who would need a more aggressive treatment. Undoubtedly, genetic factors play an important role in their origin. From the initial studies in candidate gene we have gone to the study of functional pathways in order to have a wide vision of the complex physiopathology of the cardiovascular diseases. Subtle modifications in several genes, environmental factors and the interaction between all of them may be the responsible for the development of complex diseases[1] . Therefore the study of genetic polymorphisms is an important tool to try to unravel the genetic susceptibility of the cardiovascular diseases with complex heritability patterns[2]. Nowadays the development of new microarrays, which are able to interrogate hundreds of thousands of single nucleotide polymorphisms (SNPs), and powerful tools for the analysis of the huge amount of data obtained with them, have given us the possibility of study the whole genome (http://www.affymetrix.com/products/application/clinical_research.affx).

2. CURRENT KNOWLEDGE ABOUT MICROALBUMINURIA

2.1. Definition and clinical relevance

There are large amount of information for microalbuminuria as a prognostic marker for cardiovascular and/or renal risk in diabetic and non-diabetic subjects[3-13]. Although determinants of subtle increases in urinary albumin excretion (UAE) and its progression remain poorly understood, microalbuminuria assessment is now recommended in a risk stratification strategy for hypertension management[14, 15]. Moreover, the potential of microalbuminuria as an intermediate endpoint during antihypertensive treatment is still unclear.

Microalbuminuria is defined as UAE from 30-300 mg/24 hour or equivalent amounts using timed-overnight or spot urine samples. The definition comes from studies which have established its value as a marker of risk to develop nephropathy in diabetic subjects. When the potential prognostic value of microalbuminuria on cardiovascular disease was being assessed in both diabetic and non-diabetic populations, the threshold value pointing to an increment of risk was largely below the UAE value of 30 mg/24 hour regardless of the population studied[16] . Dammsgard[5] in an elderly population demonstrated that subjects with timed-overnight UAE >7.5 mg/min had a higher mortality rate than those with lower values. Borch-Johnsen[8] in a population-based cohort of 2,085 consecutive subjects, the relative risk of ischemic heart disease associated with an spot urine albumin/creatinine ratio of only >0.65 mg/mmol, was 2.3 when adjusted for other risk factors, Likewise, Jager in the Hoorn study[9] , a population-based cohort aged 50 to 75 years followed prospectively for 5 years, albumin/creatinine ratio >2.0 mg/mmol in a spot urine was associated with a 4-fold increase in cardiovascular mortality and about an 2-fold increase in all-cause mortality. Furthermore, in a cohort of postmenopausal women living in Utrech[10] , the cardiovascular age-adjusted mortality rate for hypertensive women who were in the highest quintile of UAE was 4.3 times greater than that observed in women without detectable UAE. The highest quintile corresponded to an albumin/creatinine ratio >2.41 mg/mmol. Finally, in the cohort of subjects included in the HOPE study[11] , compared with the lowest quartile of albumin/creatinine ratio the relative risk of the primary end point in the fourth quartile, defined as albumin/creatinine ratio >1.62 mg/mmol, was 1.97.

Klausen et a[17] have delved deeply at this point, not only in looking for a threshold for risk, but also in calculating risk along a wide range of UAE. They have observed that hypertensives with a timed-overnight UAE above 5 mg/min have an increased risk for total mortality and coronary heart disease. The risk of coronary heart disease and mortality significantly increases 70% and 50% respectively when the UAE is between 5 to 10 mg/min, and 100% for both when the UAE is higher 10 mg/min. In their study, this increment of risk is independent of the factors which are known to

influence the presence of microalbuminuria: age, blood pressure levels, smoking, body mass index, diabetes, creatinine clearance or total or HDL-cholesterol. Even though UAE was only measured once, it was done in timed overnight samples which are among the most reproducible.

Two recently published studies have also shed light on our knowledge of microalbuminuria in hypertension populations and how it can be managed[18] . In one study by our group, subjects with an initial UAE level in the high normal range, from 15-29 mg/24hour, have an increased risk of progressing towards microalbuminuria. The development of microalbuminuria is linked to insufficient BP control and to a progressive increment of glucose values. Thus, appropriate intervention may reduce the progressive increment of UAE. Ibsen et al[19] in the LIFE study find that baseline and in-treatment levels of albuminuria are powerful predictors for subsequent cardiovascular morbidity and mortality. Reduction in albuminuria during treatment translates into a reduction in cardiovascular events. Although the units by which UAE have been expressed differ among the three studies, the 5 mg/min from Klausen[17] is close to the 15 mg/24 hour from our study in which there was an increased risk for UAE to increase over time, and to the 1 mg/mmol of creatinine in a morning urine spot corresponding to the second strata of risk in the Ibsen study[19].

Until studies specifically designed to answer whether or not UAE may be used as an intermediate endpoint to monitor the success of a reduction in cardiovascular and renal risk are available such in diabetes[20] , how can this new information influence daily clinical practice?. The three studies pointed to monitoring of albuminuria as an integrated part of the management of hypertension.

In both diabetic and non-diabetic subjects, the continuous relationship between UAE and cardiovascular (CV) risk raises the question of the value of UAE where there is a substantial increment of risk and, consequently, where intervention is justified. Furthermore, what should be the UAE goal during intervention?. Defining the risk of microalbuminuria at an early stage, such as at the threshold established in the Klausen study, would be adequate for guiding therapies aimed to preventing progression of UAE. If albuminuria is not decreased by a patient's current antihypertensive and/or other treatment, further intervention directed toward blood pressure control and other modifiable risks should be considered.

Finally, Klausen et al propose in the title of their article "*a new definition of microalbuminuria in hypertensive subjects*". The use of the term microalbuminuria as defined by different values according to the disease considered, eg, diabetes or hypertension, diseases which are closely linked to each other may be misleading. The use of UAE, avoiding categorization with a given threshold, should be encouraged.

The assessment of subtle increases in UAE is a powerful way to identify those at risk for multiple cardiovascular risk factor intervention. Changes in UAE seem to run in parallel to cardiovascular risk, and prompt intervention to avoid the progressive increment of UAE may result in better

protection against hypertension-induced morbidity and mortality. Although some pieces of information remain to be found, UAE has come of age in the arena of hypertension.

The next table summarizes the current criteria to define microalbuminuria according to urine samples.

Table 2. 1. *Criteria to define microalbuminuria according to urine samples.*

	Urine Sample		
Units	**Spot**	**Timed-overnight**	**24 hour**
mg/24 hour			**30-299**
mg/min		**20-199**	
mg/mmol Cr	**3-29**		
mg/mg Cr	**30-299**		

2.2. Physiopathology of microalbuminuria

Whether UAE in hypertension is mainly from glomerular or tubular origin has been studied by searching simultaneous urinary excretion of albumin and enzymes present in tubular cells, mainly beta-2-microglobulin and n-acetyl-glucosaminidase, which move to urine when tubular dysfunction is present. As suggested by the finding of normal urinary excretion of beta-2-microglobulin in normoalbuminurics and microalbuminurics with essential hypertension[21] , microalbuminuria in essential hypertensive patients is probably the consequence of an increased transglomerular passage of albumin rather than the result of a decrease in the proximal tubule reabsorption of albumin.

Increased transglomerular passage of albumin, derived from the glomerular structures in essential hypertensive patients, may result from hemodynamic-mediated mechanisms and/or functional or structural impairment of the glomerular barrier.

2.2.1. Hyperfiltration

Hyperfiltration and an increased filtration fraction have been regarded as the forerunners of haemodynamic abnormalities that may lead to the development of renal damage in primary hypertension. The presence of hyperfiltration has been considered an early sign of organ damage, and an association between an increased glomerular filtration rate (GFR) and microalbuminuria has been reported by several authors[22, 23] . There have been conflicting results, however, on the possible role of hyperfiltration and reports of either, increased[24] , unchanged[25], or decreased[26] creatinine clearance (CC) have been reported in microalbuminuric hypertensives. Minram did not find differences in basal GFR, effective plasma flow and filtration fraction between essential hypertensive patients with or without microalbuminuria[25] . Furthermore, this author observed that in a group of normal-weight, essential hypertensive subjects with hyperfiltration, urinary albumin excretion was similar to that observed in a group with normofiltration[27] . Probably the discrepancies found in these studies have reflected the unpredictable mix of functional and structural alterations leading to microalbuminuria in hypertensive patients.

Hyperfiltration, which increases glomerular pressure, is probably mediated by progressive loss of functioning nephrons and/or from abnormal transmission of systemic hypertension to the glomerulus through a disturbance in glomerular auto-regulation. Systemic blood pressure elevations are usually accompanied by increases in pre-glomerular vascular resistance preventing the increased systemic blood pressure from being transmitted to the glomeruli. The increased glomerular pressure would contribute to increase UAE, glomerular injury and progression of chronic renal failure (CRF). From clinical studies, we are now aware that alterations for constricting

afferent arterioles are present in subsets of hypertensive subjects, such as blacks, CRF, diabetics and the elderly. The prevalence of microalbuminuria in these subgroups, that have the highest risk of developing renal insufficiency, was higher than that observed in essential hypertensives. Thus, glomerular hypertension contributes to the appearance of microalbuminuria and could be a marker of risk for developing renal insufficiency among patients with essential hypertension.

2.2.2. Glomerular basal membrane abnormalities

Presence of ***glomerular basal membrane abnormalities*** as the main mechanism underlying the presence of microalbuminuria in hypertension has been suggested by several authors. Other studies, however, did not support this point of view. Urinary transferrin excretion (UTE), a more anionic and higher size molecule than the albumin molecule, was studied simultaneously to the UAE in hypertension. In contrast to observations in diabetic subjects, in which a correlation between UTE and UAE was present[28] , no relationship between UTE and UAE was observed in hypertensives, and the impact of blood pressure changes in UTE was negligible[29] .

2.2.3. Endothelial dysfunction

Microalbuminuria reflect the kidney expression of a more generalized state of endothelial dysfunction [30] . Higher levels of circulating von Willebrand factor antigen, a glycoprotein released in greater concentrations when endothelial cells are damaged, were found in microalbuminuric compared to well-matched normoalbuminuric patients with essential hypertension. A direct correlation between von Willebrand factor antigen and albumin excretion rate was described, supporting the hypothesis that albuminuria reflects systemic dysfunction of the vascular endothelium[26].

2.2.4. Nephrosclerosis

Nephrosclerosis needs to be mentioned when abnormal UAE in essential hypertension is the issue. Glomerular and vascular structural alterations coexist with altered functional mechanisms particularly in the more severe cases and in those with long-standing disease, but in most patients with primary hypertension and microalbuminuria no specific pathologic kidney changes can be found[31] . Studies of the natural history of nephrosclerosis in essential hypertension are lacking, due to the limitation of carrying out renal biopsies on these patients. Data from experimental rat models, such as streptozotocin-induced diabetes and 5/6 renal ablation, induces albuminuria early after injury which progresses throughout the observation period. Micropunture studies performed

during the initial post-injury phase, in a normotensive state, showed the presence of hyperfiltration, and an increase in intraglomerular capillary pressure resulting from preferential dilatation of the afferent glomerular arteriole. Later, glomerular hypertension induces a progressive nephron loss. Proteinuria appears from the beginning supporting the role of hyperfiltration in the increment of urinary albumin excretion. Moreover, the use of micropuncture techniques has shown that albuminuria originates from intact glomeruli rather than damaged ones[32] . Whether an increase in the transglomerular passage of albumin is a cause or a consequence of glomerulosclerosis remains a subject of debate.

2.3. Factors related to microalbuminuria

Factors related to the presence of microalbuminuria in essential hypertension have been analyzed in cross-sectional studies, but follow-up studies are rare[7, 33-35]. From these studies it seems that the significance of microalbuminuria in essential hypertension is much broader than expected, and several factors may influence the presence of microalbuminuria. An overview of these is presented in the following sections.

2.3.1. Cross-sectional studies

During the last decade, cross-sectional studies have tried to analyze what the determinants of microalbuminuria in essential hypertension are. Although there are several determinants which appear important in all the studies, others depend on the selection of the study population and on the interest of the authors vis a vis specific factors. Subsequently, microalbuminuria has been related to blood pressure (BP) values[36-39] and to hyperinsulinemia as an expression of insulin resistance[40, 41].

2.3.1.1. Blood pressure values

Blood pressure is the main determinant of microalbuminuria in both diabetics as well as in non-diabetics. A positive relationship between blood pressure levels obtained in the office and UAE in hypertensives was observed in several studies[36-39] . The impact of BP values on the presence of microalbuminuria has been further reinforced by data coming from studies using 24-hour ambulatory BP monitoring (ABPM). Ambulatory blood pressure values showed a stronger association with UAE than office blood pressure measurements did[42-44] . In one study by our group, conducted on patients with essential hypertension, significant differences in blood pressure were found between microalbuminuric patients and normoalbuminurics[44] . The differences were maintained throughout the 24-hour data collection period, reflecting that ambulatory blood pressure measurements were better predictors than casual measurements were for the presence of microalbuminuria.

A blunted physiological nocturnal BP fall, and then the persistence of elevated BP during night, were also associated with high UAE rates[44] . Providing further evidence of the importance of blood pressure during night, patients with a lower day/night ratio had higher UAE even when controlling for daytime blood pressure. This observation could be interpreted as high blood pressure maintained over time leading to an increase in UAE.

Blood pressure leads to endothelial dysfunction resulting in an increase in the permeability of macromolecules and the glomerular capilar wall does not escape this impact. The higher the blood pressure the higher the endothelial dysfunction. In addition, persistence of high BP levels

during the night-time implies that during the recumbent position there is a reduced activity of the mediators controlling pre-glomerular tone, mainly sympathetic and renin-angiotensin activities. The presence of conditions that impair, and thereby reduce, pre-glomerular tone permit further and easier transmission of high pressure into the glomeruli which trigger mechanisms increasing the UAE[45].

2.3.1.2. Insulin resistance and hyperinsulinemia

Hypertensive subjects with microalbuminuria have shown higher values for fasting insulin and/or insulin after glucose challenge[40, 41], and hyperinsulinemia has been related to microalbuminuria both in hypertensive subjects as well as in non-diabetic normotensives. In one study by our group[41] , we found higher values for both fasting insulin and area-under the curve (AUC) of insulin during an oral glucose-tolerance test (OGTT) in the microalbuminuric group when these factors were compared to those of the normoalbuminuric one.

Hyperinsulinemia is probably an expression of insulin resistance, and a higher degree of peripheral resistance to insulin, estimated by euglucemic-clamp, has been described in microalbuminuric patients with non-insulin dependent diabetes mellitus[46, 47] or hypertension[48].

The link between hyperinsulinemia and microalbuminuria seems to be recognized in several studies, and microalbuminuria has been included as a component of the metabolic syndrome. Insulin may lead to the increase in UAE enhancing the endothelial permeability of albumin in the kidney. In healthy subjects, an increase in the transcapillary escape rate of albumin produced by insulin infusion has been described[49] . Whether a direct effect of insulin in the endothelial permeability of albumin is present or not, other factors, such as the impact on concurrent factors such as glomerular hemodinamics, salt balance, and/or overactivity of angiotensin II and sympathetic nervous system, might also contribute to the proalbuminuric effect of insulin.

2.3.1.3. Salt-sensitivity

Some observations have linked salt-sensitivity to microalbuminuria in essential hypertension[40, 50], and salt-sensitivity may be a prevalent state among microalbuminuric hypertensives. Salt-sensitive hypertensive subjects manifest complex abnormalities: an exaggerated BP response to noradrenaline and angiotensine II, reduction in urinary kallikrein excretion, decreased insulin sensitivity, and renal hemodynamic derangement[51]. In salt-sensitive hypertensive patients, even small increases in dietary salt may have potentially detrimental effects on glomerular hemodynamics and proteinuria[52] . The corollary is that the presence of more UAE in salt-sensitive patients could be interpreted as a marker of greater renal damage, and potentially as a prognostic indicator of the future progression of the renal disease. Indeed, a

modest salt reduction may be helpful by reducing the risk of adverse renal hemodynamic changes in the subjects who display microalbuminuria.

2.3.1.4. Overactivity of the Renin-Angiotensin System

The hypothesis that microalbuminuria can be a marker of more severe renal ischemia and activated renin system was supported by the study of Erley and cols[53] , who found higher postcaptopril plasma renin activity in young patients with essential hypertension who had albuminuria than in those without. Moreover, an increased albumin excretion rate in essential hypertensive patients with *DD* genotype of the *I/D* polymorphism of the angiotensin converting enzyme (ACE) has been described[54] . This genotype is characterized by high serum levels of ACE and possibly of angiotensin II, both at circulating and tissue levels.

2.3.1.5. Inflammation

The impact of low-grade inflammation in the UAE has been analyzed in several studies[55] . Although both C-reactive protein (CRP), a marker of low-grade inflammation in the vascular wall, and microalbuminuria cluster frequently, the positive relationship between serum C-reactive protein and microalbuminuria emerges mainly in subjects in absence of other major cardiovascular risk factors such as hypertension and/or diabetes [56, 57]. In essential hypertension, however, levels of CRP modulate the relation between blood pressure and UAE, and for a given BP value the higher CRP the higher UAE [58]. A potential explanation for these observations is that low-grade inflammation is not a strong determinant of microalbuminuria, but inflammation can booster the impact of other strongest factors such as high blood pressure levels.

2.3.2. Follow-up studies

Long-term studies of microalbuminuria in essential hypertension are rare[7, 33-35], and information about the contribution of factors other than BP reduction in the changes of UAE overtime are scarce. Again in these studies the potential risk factors are similar to those which were analyzed in cross-sectional studies, although others deserve attention.

2.3.2.1. Blood pressure and glucose values

Blood pressure role has been analyzed prospectively, mainly in patients with diabetes. The normotensive arm of the ABCD study in Type 2 diabetes has demonstrated that patients with an

intensive diastolic BP control program, 128/75 mmHg, had a lower risk of developing microalbuminuria than those with a moderate therapy, 137/81 mmHg did[59]. Furthermore, a progressive increase in systolic BP during sleep has been related to the development of microalbuminuria in normotensive Type 1 diabetics[60].

In patients with essential hypertension, several studies demonstrated that by reducing BP values the UAE decreases significantly, mainly when the BP reduction was large enough. In other cases, when the BP reduction is small, antihypertensive drugs blocking the activity of the renin-angiotensin system seem to be more beneficial than in the other antihypertensive groups[61]. All of these studies have focused on the BP changes and not on other potential changes.

Our group conducted a prospective study to assess factors related to the occurrence of microalbuminuria during the follow-up of a group of never previously treated young adults with essential hypertension[35] . The main factor influencing the occurrence of microalbuminuria during antihypertensive treatment was the slope of systolic blood pressure. Thus, BP needs to be lowered as much as possible in order to prevent occurrence of microalbuminuria.

Besides the impact of SBP overtime in the risk of developing microalbuminuria, a trend in increasing glucose values was also a determinant of risk, which was independent of the BP values[18, 35].

2.3.2.2. Baseline urinary albumin excretion

The importance of the baseline level of UAE on the risk of developing microalbuminuria has been analyzed in two follow-up studies of non-diabetic essential hypertensives. Agewall et al[62] studied 213 patients without microalbuminuria after a three-year follow-up, controlled biannually, using the level of UAE from a single measurement of overnight UAE. This study observed that patients who developed microalbuminuria had higher UAE at baseline compared with those who remained normoalbuminurics. Probably, the increase in baseline UAE is, at least in part, an expression of cardiovascular factors and end-organ damage even in values below the threshold to define microalbuminuria. Redon et al[35] studied 187 normoalbuminuric subjects in whom baseline UAE was one of the determinants for the risk of developing microalbuminuria.

The following table summarizes those factors associated to the prevalence (cross-sectional studies) or the incidence (follow-up studies) of microalbuminuria in essential hypertension.

Table 2. 2 . *Factors associated to the prevalence or the incidence of microalbuminuria in essential hypertension.*

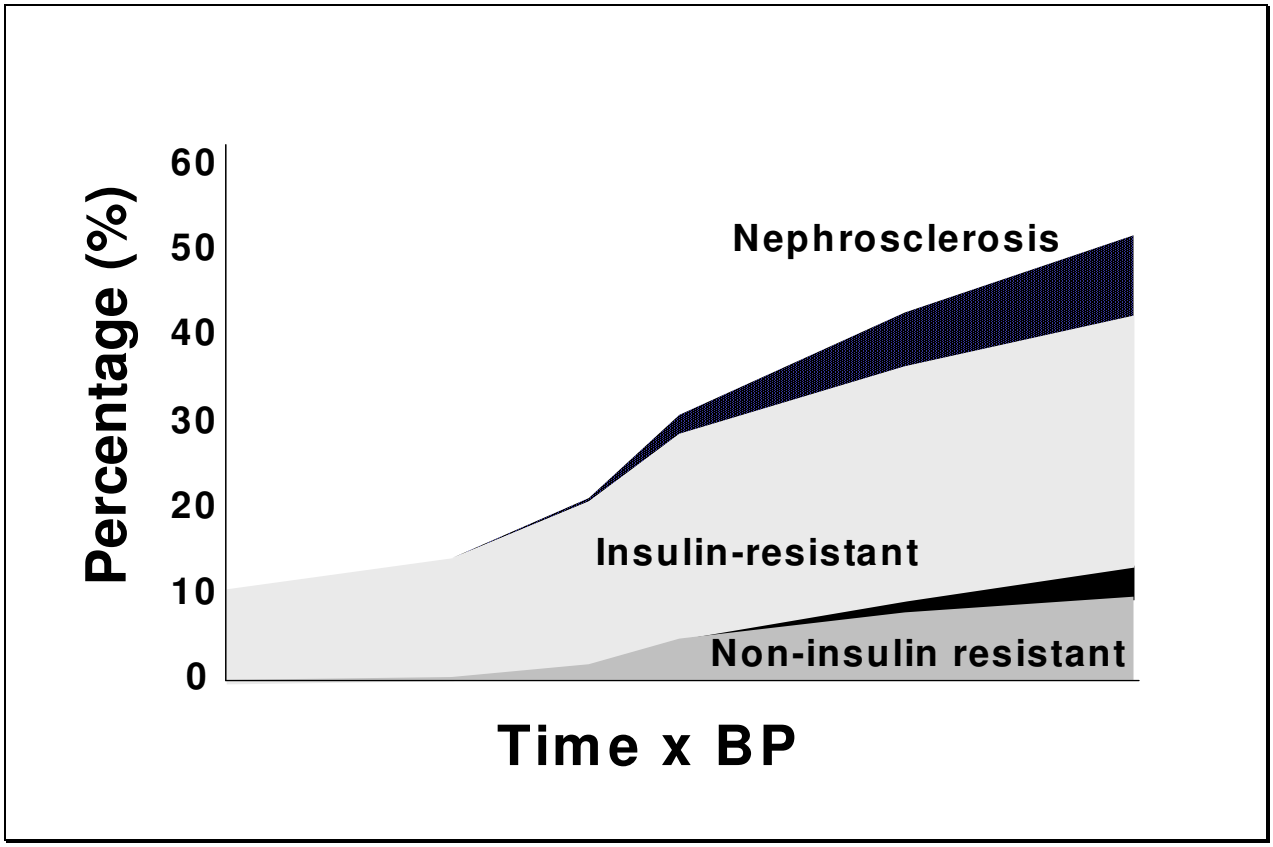
Variable	Prevalence	Incidence
Baseline UAE	NA	+++
BP values	+++	+++
Glucose values	+	++
Hyperinsulinemia	+++	NA
Genetics	+	+
Body weight	+	+
Smoking	+	NA

NA: not available

2.4. Natural history of microalbuminuria in hypertension

Considering information available, we can hypothesize and graphically represent the natural history of microalbuminuria in hypertension (Figure 2.1).

Figure 2. 1. *Natural history of microalbuminuria in hypertension.*



In hypertensive subjects microalbuminuria may be the consequence of a double product, time of hypertension per blood pressure values. If the patient has insulin-resistance, microalbuminuria can be present even when the double product of time and pressure is small. By contrast subjects without insulin-resistance need a long time and/or high blood pressure values to develop microalbuminuria. At the top of these scenarios, the development of nefroangiosclerosis, less prevalent in non-insulin resistance, adds a new component to the risk of having microalbuminuria.

2.5. Effects of therapy in microalbuminuria

2.5.1. Short term effects

A substantial decrease in microalbuminuria as a result of the effective control of hypertension is suggested by the correlation between blood pressure levels and microalbuminuria. Lowering blood pressure with antihypertensive medication reduces albuminuria in diabetes[63] and in primary hypertension[61] according to several studies. The reduction has been observed when using agents as diverse as angiotensin-converting enzyme inhibitors (ACEi), angiotensin II-receptor blockers (ARBs), betablockers, calcium channel blockers or a combination therapy. Questions have arisen over whether antihypertensive agents differ with respect to their action on microalbuminuria, and whether hypertensive patients do not reduce or whether they even increase UAE despite blood pressure control.

At the same level of achieved BP, antihypertensive drugs blocking the activity of the renin-angiotensin system, both angiotensin converting enzyme inhibitors (ACEi) and angiotensin II receptor antagonists, seem to be more beneficial than the other antihypertensive groups[61, 63]. The ACEi and angiotensin II blocking-receptors (ARBs) have additional beneficial effects on microalbuminuria, independent of BP reduction, since they reduce the biological actions of angiotensin II. It has been implicated in the progression of renal failure in diabetic and non-diabetic nephropathy, by up-regulating the expression of growth factors and cytokines such as transforming growth factor-beta1, tumor necrosis factor-alpha (TNF-α), osteopontin, vascular cell adhesion molecule1, nuclear factor-kappa, platelet-derived growth factor, fibroblast growth factor and insulin-like growth factor[64, 65]. ACEi and ARBs reduce the increased levels of some of these growth factors and cytokines[65].

Some research, however, has reported discordant results. Erley and coworkers[66] , who used felodipine, doxazosin, ramipril and atenolol noted a similar lowering of blood pressure and a decrease of UAE. Discrepancies between studies like of Erley´s one and others may be explained by the initial blood pressure values, the extent of blood pressure fall during treatment and the presence of factors other than BP which determine UAE, glucose metabolism abnormalities and salt intake.

The beneficial effects of the antihypertensive agents on microalbuminuria are proportional to BP reduction. The higher the initial blood pressure the greater the UAE reduction. If a large enough BP reduction is achieved, differences among the antihypertensive drug classes diminish.

Glucose metabolism derangement induces microalbuminuria even though BP values are “quasi” normal since hyperfiltration and basal membrane alterations have been described as a consequence of hyperglycemia. Despite lowering BP, no change or only a minimal reduction in UAE occurs.

Dietary salt intake is a missed factor in the majority of the studies. Salt influences UAE through its impact on the activity of the renin-angiotensin system and on BP values. There is a high prevalence of salt-sensitive subjects in the subset of hypertensives who are microalbuminuric, and changes in salt-intake significantly modify BP, glomerular hemodynamics and consequently, UAE[52]. Until more information is available, patients with microalbuminuria should be encouraged to moderate their salt intake.

The regression of UAE has been observed shortly after BP reduction, favouring the hypothesis that the regression comes from a reduction of albumin filtration in the glomerulus as a consequence of a lower intraglomerular pressure. Hypertensive patients with persistent microalbuminuria under short, intensive antihypertensive therapy, however, show a statistically significant higher prevalence of hypertensive retinopathy and, therefore, microalbuminuria can be considered an indicator of general microvascular damage in essential hypertension[67].

In summary, the heterogeneity in the UAE changes during antihypertensive treatment may be related to the different factors involved in the presence of microalbuminuria and/or to the presence of structural end-organ damage. Short-term UAE reduction with antihypertensive treatment depends on initial BP, extent of BP reduction, antihypertensive class, factors other than BP and structural abnormalities established in the glomerulus.

2.5.2. Long term effects

Long-term studies of microalbuminuria in essential hypertension are rare [7, 19, 34, 35], and information about the contribution of factors other than BP reduction in the changes of UAE over time is scarce. The information available comes from studies in which changes in UAE over time were presented as a whole or separately for subjects with or without response to treatment.

In the LIFE study[68], yearly examination of UAE has performed in a cohort of essential hypertensives with left ventricular hypertrophy and the median changes in albuminuria across 5 years of follow-up has reported. At each year the decrease in albuminuria is more pronounced for losartan-based as compared with atenolol-based treatment[68]. At the first and second year of follow-up, a 33% reduction was seen for losartan as compared with 25% for atenolol. By observing the reported data, the initial reduction is progressively attenuated over time. After the initial reduction, which is greater for losartan as compared to atenolol, the trend toward the initial levels seems to be similar for both groups of treatment[68]. This means that in hypertensive subjects as a

whole, UAE increases progressively despite the antihypertensive treatment. Whether or not this is the result of calculating the average of UAE from subjects increasing and decreasing it is not offered by the authors. Another potential explanation for the observation in the LIFE study is that the BP achieved during treatment, 144/84 mmHg, is too high to protect against the increment in UAE.

The Prevention of Renal and Vascular End-Stage Disease Intervention Trial (PREVEND-IT)[69] cohort of approximately 800 otherwise healthy subjects with high levels of urinary albumin have selected out of the PREVEND general population cohort. They have randomized to receive the ACEi fosinopril 20 mg/d or placebo, and pravastatin 40 mg/d or placebo, for 4 years. After 3 months, albuminuria levels have significantly reduced by approximately 30% in patients assigned to fosinopril as compared with placebo, and the reduced levels have sustained at the 4-year follow-up.

Studies showing individual changes provide more useful information when trying to understand the evolution of UAE under antihypertensive treatment. In one study[18] , from 245 normoalbuminuric hypertensives, representative of a middle-aged population with mild hypertension, in thirty subjects (12.2%), UAE increases to the microalbuminuric range, from 13.1±7.7 to 44.2±18.2 mg/24 hours, ($p<0.001$) after a mean follow-up of 29.9 months with a conversion rate of 2.5/100 patients/year. Two hundred and fifteen subjects (87.8%) do not develop microalbuminuria after a mean follow-up of 61.7 months. In these patients, UAE do not increase even within the normoalbuminuric range (from 9.4±6.2 to 9.0±9.7 mg/24 hrs). Subjects who remained normoalbuminurics over time, maintain stable levels in sharp contrast with those who become microalbuminurics in which a progressive increase occurs.

In this study [18], in initially normoalbuminuric hypertensive subjects the persistence of higher than normal SBP precedes the development of microalbuminuria. Additionally, an upward trend in fasting glucose values seems to be present in those subjects who develop microalbuminuria. Thus, the potential for developing microalbuminuria appears very low in subjects who achieve strict normal blood pressure, or in those who maintain stable fasting glucose values.

The role of blood pressure on the evolution of microalbuminuria has been analyzed prospectively, mainly in patients with diabetes. The normotensive arm of the ABCD study in Type 2 diabetes has demonstrated that patients with an intensive diastolic BP control program, 128/75 mmHg have a lower risk of developing microalbuminuria than did those with a moderate therapy, 137/81 mmHg[59]. Furthermore, a progressive increase in systolic BP during sleep has been related to the development of microalbuminuria in normotensive Type 1 diabetics[60] . A significant increase of 2.2 times in the risk to develop microalbuminuria for SBP > 139 mmHg over the subjects with SBP <130 mmHg, is observed in one study performed in essential hypertension subjects[18] .

Besides the impact of SBP over time in the risk of developing microalbuminuria, a trend in increasing glucose values is also a determinant of risk which is independent of the BP values. The risk of developing microalbuminuria is sharply increased in patients with a positive slope of glucose plasma levels. This seems to reflect that the patients in the study prone to developing microalbuminuria are at an early stage of carbohydrate metabolism abnormality. The upward trend in glucose levels in those hypertensives who become microalbuminurics is in agreement with data coming from The Framingham Offspring Study[70] in which 24-year time-integrated fasting glucose levels are strongly associated with the risk of developing microalbuminuria in the general population. The increased risk is not limited to diabetic levels of fasting glucose, and subtle elevations in apparently normal levels also increases the risk, suggesting that the increased risk occurs in a graded fashion across the spectrum of glucose tolerance[71] . A significant increase in the risk of 2.2 times to develop microalbuminuria for a positive trend in fasting glucose as compared to those with a neutral or negative trend have been observed independent of baseline UAE and SBP values.

3. GENETIC BASES OF MICROALBUMINURIA AND RELATED TRAITS

3.1. Overview

Microalbuminuria has become a marker of risk not only for renal, but also for cardiovascular disease in diabetes, hypertension (HTN) and in the general population. In hypertension, microalbuminuria has been associated with a clustering of cardiovascular risk factors. Moreover, changes in microalbuminuria over time seem to have prognostic implications. Therefore, microalbuminuria assessment is now recommended for the stratification of risk of patients with HTN [4-13]. Although microalbuminuria reflects systemic endothelial dysfunction[26, 30], it is also a marker of renal damage that can antedate the development of proteinuria[20, 23, 31] , an unequivocal sign of established nephropathy.

Factors related to the development of microalbuminuria have been extensively analyzed in cross-sectional and follow-up studies over the last several years. Blood pressure levels, hyperglucemia, hyperinsulinemia and insulin resistance are probably the main factors[36-39]. Other factors such as obesity[72] and smoking[73] have also been implicated in the risk of developing microalbuminuria. The role of genetic factors has been considered to be based both on epidemiological, as well as on animal studies[74, 75]. Familial clustering of renal disease [76, 77] and racial differences in risk for end-stage renal disease [78-80] [81] have been recognized. Likewise, a susceptibility loci for renal disease and UAE has been identified in rats[82].

In the following pages of this doctoral thesis, a systematic review summarizing the current knowledge of the genetics of microalbuminuria and related traits in essential hypertension is presented.

3.2. Evidence supporting the influence of genetic factors for renal traits

The first line of evidence is the genetic epidemiological studies. These kinds of studies demonstrate that there is a strong familiar clustering for renal disease and microalbuminuria not only in type 1 and type 2 diabetes but also in HTN. In this way, it has been demonstrated that relatives of patients with type 2 diabetes (DM2) are prone to develop microalbuminuria even if they were not diabetics themselves and this microalbuminuria can finally drive to the end stage renal disease (ESRD)[76, 77]. Also the ESRD itself have a strong family clustering in diabetes and HTN[77, 83].

Another piece of evidence are the huge differences existing between ethnics regarding to the prevalence of renal damage. There is some ethnics which are in high risk of develop diabetic or hypertensive ESRD such as Afro-Americans or Hispano-Americans[78-80]. It is not clear if that differences between ethnics are due to environmental factors such as different lifestyle or inequalities in the availability of the treatments or to different genetic backgrounds[81].

Finally we have several studies performed in animals which strongly support the presence of susceptibility loci for renal disease. In mice, several quantitative trait loci (QTL) have been linked with renal traits including UAE[82]. Moreover, since 2005 it is available an internet tool to compare humans and mice quantitative trait loci related with renal traits (http://pga.jax.org/qtl/kidneyqtltable.htm). For UAE, in hypertensive rats, there are QTL on chromosomes 2 and 3 which correspond with human QTL for albumin creatinine ratio (ACR) on chromosomes 3 and 20 according to one linkage study in type 2 diabetics Pima Indians [84]. Besides those linkage studies, knockout mice models also demonstrate the relevance of several candidate genes, such as endothelial nitric oxide synthase (eNOS), in renal damage and UAE [85, 86].

Taking into account all of these facts, it is clear that genetic plays an important role in microalbuminuria, but what are the genes or variants involved remains unclear.

3.3. Elucidating the genetic susceptibility of microalbuminuria and renal traits

Candidate gene (CG), linkage analysis (LA) and genome wide analysis (GWAS) has been used to unravel genetic variants of susceptibility to microalbuminuria and renal damage in essential hypertension. Although detailed analysis of the advantages and the limitations of each of the methods used is out of the scope of the present doctoral thesis, some key aspects need to be acknowledged.

Candidate genes strategy, which tries to simplify the study of complex diseases by selecting of a small set of genes and then studying the associations of polymorphisms with the problem trait, has the strength of the biological plausibility. Additional functional in vitro studies and knockout mice models of the polymorphisms detected can help to assess the relevance of the association. This is the case of the polymorphisms of eNOS and of angiotensin II type 1 receptor (ATR_1) genes, which have been associated with nephroangioesclerosis[87] and UAE [85, 86]. Candidate gene studies take advantage of the detailed data base of gene SNPs, the HapMap project (http://www.hapmap.org/) and of haplotype construction by creating stronger markers. Epistatic interaction with other physiologically related genes is used to detect interactions between different components on the same metabolic pathway or on related ones.

Linkage studies within families evaluate the coincident inheritance of polymorphic genetic markers across the genome among family members with the disease status. Heritability, susceptible loci and potential candidate genes associated with a particular disease are obtained and have successfully been used in animal models of renal damage. Linkage of urinary albumin excretion with two QTL on chromosomes 2 and 3 have been described, and this linkage has been replicated in type 2 diabetic Pima Indians [84]. In general, linkage strategy has been useful for oligo or monogenic disease, but it has failed in complex diseases which must be produced by the interaction of multiple variants of low risk.

The most recent approach is the genome wide association studies (GWAS) in unrelated subjects. Covering all the genome with such a huge density of markers offers the possibility of finding previously unsuspected associations with physiological systems. The new high-throughput microarrays can contain up to more than 1 million markers across the genome, not only SNPs, but also other polymorphisms such as copy number variation (CNV). The GWAS have been shown to be useful for some complex diseases such as types 1 and 2 diabetes, bipolar disorder or coronary heart disease, but in HTN the initial results were negatives.[88-91].

Although a combination of different strategies can reap benefits from the advantages of each of them and help refine the studies, there are several problems common to all genetic studies. Sample size, marker density, genotype quality and phenotype selection need to be adequately identified to minimize the risk of false associations.

Among the phenotypes of renal disease in HTN, *Renal insufficiency* (RI), assessed by plasma creatinine values or estimated GFR (eGFR), and ESRD are easy to appraise, although ESRD takes a long time to develop and its incidence is rare. *Nephroangiosclerosis*, the renal damage induced mainly by HTN, is a misleading trait because its diagnosis using clinical criteria can be erroneous in the absence of renal biopsy. Consequently, UAE has been the most widely used marker of renal damage in HTN. Proteinuria as a qualitative trait has been used, but its incidence and prevalence are low in HTN. Microalbuminuria, a small amount of UAE, is much more frequent and has been used as either a quantitative trait or a qualitative one when UAE is within the range 30-300 mg/day. Whether using the quantitative or the qualitative approach, the within individual variability needs to be taken into account so more than one sample is necessary to achieve accuracy in the assessment. Quantification of UAE in a twenty-four hour urine sample, nocturnal timed-urine collection or albumin/creatinine ratio in spot morning urine is used. Assessing the temporal trend of UAE, in order to gain precision at the time of qualifying the trait, has also been used in a few studies.

3.4. Genetic bases of microalbuminuria and related traits in essential hypertension and general population

3.4.1. Candidate gene studies

The candidate genes analyzed in association studies with renal traits in HTN have pertained to those codifying proteins or peptides of the renin-angiotensin-aldosterone system (RAAS), natriuretic peptides, the adrenergic system, inflammation, oxidative stress, intracellular signalling, lipid metabolism, regulation of the extracellular matrix, fibrinolysis among others.

A resume of some of the candidate gene studies for renal traits in essential hypertension is shown in table 3.1 (I,II,III).

3.4.1.1. RAAS genes

The genes of the RAAS are among the most studied. This physiological pathway regulates the plasma and tissue levels of angiotensin II, a peptide which is involved not only in glomerular, but also vascular damage, and therefore in the development of HTN-induced nephroangioesclerosis[92-94]. Moreover, drugs blocking either the generation of angiotensin II or the binding to the AT1- receptor are able to reduce UAE beyond the BP lowering effect and, therefore, protect the kidney [95-99] .

The *Insertion/Deletion polymorphism* (*I/D*) of the angiotensin converting enzyme (ACE) gene, *M235T* of the angiotensinogen (AGT) gene, *A1166C* in the angiotensin II type 1 receptor (AT1R) gene, and *T344C* of the aldosterone synthase (CYP11B2) are among the gene variants which have been associated with renal traits.

The *D* allele of the *I/D* polymorphism in ACE seems to increase the risk of nephroangioesclerosis independent of BP levels. One retrospective study found a significantly higher prevalence of the *DD* genotypes in patients with nephroangioesclerosis diagnosed by biopsy compared with hypertensive patients without renal damage[74], a finding replicated in an independent Italian cohort of hypertensive patients with or without renal damage[100] .

The risk of RI in one cross-sectional study in an Italian population found an increase of risk of having renal failure (Cr>1.5 mg/dl) for carriers of various genotypes: *DD* in *I/D* of ACE, *TT* in *M235T* of AGT, *CC* in *A1166C* of AT_1R, and *CC* in *C344T* of CYP11B2[101] . The strongest risk was for the *TT* genotype of the *M235T* polymorphism in the AGT gene[101]. The study also found certain epistatic effects among these variants. Moreover, the combination of the opposite alleles showed a protective role for renal damage[101].

Concerning the association with microalbuminuria, in one cross-sectional study carried out in a subset of never before treated young hypertensives, there was a trend to present higher levels of UAE in carriers of the *DD* genotype of *I/D* in the ACE gene than for the other genotypes[102]. Furthermore, the UAE in these patients showed a stronger relationship with BP levels than it did for the other genotypes [102]. In a prospective study, patients with the *DD* genotype showed a significant positive correlation between the slopes of SBP and UAE (r=0.60) over time[75]. This correlation was not present in carriers of the *I* allele. Some findings[75, 102] suggest that this polymorphism can modulate the impact of the blood pressure in kidney damage.

Two polymorphisms of the AT_1R gene (*A1166C* and *C573T*) were also tested in a subset of 183 hypertensive patients less than 50 years old[103]. The *TT* genotype of *C573T* had significantly lower levels of UAE than did the other genotypes[103]. A similar and significant result was found for the *AA* genotype of *A1166C*, as this allele *A* is closely linked with the allele *T* of the *C573T* polymorphism[103].

In the AGT gene, the *M235T* and another polymorphism in strong linkage disequilibrium, *A-6G*, located in the promoter region in a prospective study in young hypertensives have also been studied [104]. Although no significant differences in genotypes for either polymorphism was observed between patients with or without microalbuminuria, the *A-6G* polymorphism seems to influence the risk of new onset microalbuminuria in a three year follow-up study[104]. In this case, the development of microalbuminuria was linked to the changes in fasting glucose and weight[104, 105], a previously described association with *M235T*.

In the study performed by Pontremoli et all[106], patients carrying the allele *D* in ECA, *T* in AGT and *C* in AT_1R have higher early organ damage as compared to carriers of the other alleles of the polymorphisms studied. These were not only associated with the greatest UAE, but also with left ventricular hypertrophy and increased intima media thickness (IMT).

3.4.1.2. Natriuretic peptides and alpha-adducin genes

The human atrial natriuretic peptide (ANP) plays a key role in the volume handling, and interaction with the RAAS system influences renal haemodynamics[107-109]. The ANP gene has been associated with microalbuminuria and renal impairment. The mutant allele A^1 of the *2238T>C* polymorphism, also called the *ScaI*[110] polymorphism, which is a restriction fragment length polymorphism (RFLP), seems to be less frequent in microalbuminuric hypertensive patients with diabetes according to one Italian study[111]. This allele A^1 causes a break in the stop codon, which results in an ANP with two more arginine molecules, and could protect against renal damage. The functional hypothesis is that this variant reduces the blood levels of ANP and, therefore, the renal damage induced by hyperfiltration. In the same study, another mutant allele, *T*, of the *708C>T* (*rs5064*) polymorphism was less frequent in subjects with

microalbuminuria[111]. In a partial replication only the *Scal* polymorphism, but not the *T* allele in diabetics, was observed in a Mexican population[112].

Also related to sodium handling is the Alpha-Adducin (ADD1) gene. The *Gly460Trp* polymorphism of this gene has been related to salt sensitivity and several renal traits[113, 114]. Regarding microalbuminuria, one Italian study failed to find an individual association, although an epistatic interaction with the *I/D* polymorphism in the ECA gene was associated with increased risk of microalbuminuria[115]. Homozygotes *DD* for the *I/D* polymorphism in ECA and *460Gly* of the ADD1 gene had greater prevalence of microalbuminuria than carriers of the *I* allele did. A controversial result was observed in another study in which the allele *460Try* in the ADD1 gene seems to interact with the *D* allele of the *I/D* polymorphism in ECA gene to increase the risk of mild renal dysfunction[116].

3.4.1.3. Adrenergic system and adrenomedulin genes

The adrenergic system plays an important role in the development of hypertension, obesity, insulin resistance and, therefore, with hypertension-induce organ damage[117-119]. Overactivity of the sympathetic nervous system (SNS) may be one of the contributing factors to the development of UAE and renal impairment[120, 121] .

Masuo et all[122], in a 5 year follow-up study including nonobese normotensive patients with normal renal function, found a relationship between high levels of norepinephrine at the beginning of the follow up, the allele *Gly16* of the *Arg16Gly* in the beta/adrenergic receptor type 2 gen (ADRBR2) and the decrease of renal function over time. Polymorphisms of the ADRBR2 have also been related with left ventricular remodelling and its regression after hypertensive treatment [123]. Among the three polymorphisms studied, *Gly16Arg, Gln27Glu*, and *Thr164Ile*, only the allele *27Glu* of the *Gln27Glu* was associated with a larger cardiac size at baseline and with a better response to the enalapril treatment than those carrying the other allele *Gln27*[123]. Interestingly, the associated SNP was in strong linkage disequilibrium with *Gly16Arg* which has shown a relationship with renal impairment over time in nonobese normotensive patients[122].

Related to the adrenergic system is the gene which codifies adrenomedulin. Patients with cardiovascular disease may have an increase in their blood levels of adrenomedulin[124], a hormone which is mainly secreted by endothelial cells and has important functions in the cardiovascular system including diuresis, vasodilatation, aldosterone inhibition and cardiac output regulation[125-127]. Kobayashi Y et al[128] analyzed several polymorphisms and haplotypes of the adrenomedulin gene in a Japanese hypertensive population. They were unable to find an association with HTN, but some haplotypes, including allele *19* for the number of repeats in

the microsatellite, the *A* allele of the SNP *rs4399321* and the *G* allele of the SNP *rs7944706*[128] were associated with microalbuminuria[128].

3.4.1.4. Oxidative stress

The endothelial nitric oxide synthase (eNOS) is another gene that studies have tried to associate with renal impairment and microalbuminuria in HTN. The results are conflicting. One of these studies, carried out in an Italian population with HTN, did not find an association between eNOS polymorphisms and either microalbuminuria or endothelial dysfunction[129]. Another study tried to relate two polymorphisms of the eNOS gene with ESRD of several etiologies. Although the study only included a small number of ESRD due to HTN, the *tandem repeat* polymorphism seems to influence the ESRD in non-diabetic subjects, whereas the *Glu298Asp* polymorphism could be related to ESRD of any etiology[130].

3.4.1.5. Kallikreins

Genes which codify kallikreins can also be related to hypertension-induced renal damage. Urinary excretion of kallikreins may have a genetic component and have been previously associated with renal impairment and HTN[131, 132]. The tissular form of kallikrein depends on genes which are located in cytoband q13.3 of chromosome 9 whereas the plasmatic form is codified by genes on chromosome 4q34-35[133, 134].

Polymorphisms in the promoter region of the kallikrein 1 gene were studied in order to assess the possible association with ESRD in African-American subjects[135] The allele *12G* of the *-130(G)N* polymorphism was associated with ESRD, but only in the subgroup of hypertensive patients[135]. There was no association in the group of diabetics nor was there when diabetics and hypertensives were compared jointly against the control group[135].

3.4.1.6. Other genes

Another interesting gene is the non-muscle myosin heavy chain 9 gene (MYH9) which has been associated with segmental and focal glomerulosclerosis (FSGS)[136, 137]. It seems also to be associated with renal damage associated with hypertension, at least in African-American populations. Within the HyperGen study population, several SNPs of the MYH9 were associated with the albumin/creatinine ratio after adjusting for confounding factors, both in unrelated African-Americans, as well as within African-American families. In contrast, there was no association within European-American families[138]. Moreover, one polymorphism and one haplotype of this gene showed an stronger association with ESRD due to HTN than that for albumin/creatinine ratio [139]. The fact that this gene is expressed in podocytes and involved in segmental or global glomerulosclerosis seems to give credit to its role as a candidate gene

for hypertension-related glomerulosclerosis. What is not clear is if the hypertension is due to the renal disease or vice versa.

Table 3.1. *Resume of candidate gene studies in HTN (I).*

Author, year (ref)	GENE	POPULATION	SAMPLE SIZE (Cases/Controls)	STUDY DESIGN	GENE/POLYMORPHISM	RISK ALLELE	MODEL	Results
RAAS system								
{Fernandez-Llama, 1998 #74}	*Angiotensin converting enzyme (ACE)*	Hypertension Caucasian	37/75	Retrospective (Case/Control)	ACE/I/D	D	Co-dominant	DD genotype was associated with nephroangiosclerosis diagnosed by biopsy
{Rovira, 1999 #102}	*Angiotensin converting enzyme (ACE)*	Hypertension Caucasian	79	Cross-sectional (Quantitative trait)	ACE I/D	D	Co-dominant	Trend to present higher levels of UAE for DD genotype than the carriers of I allele; Significant interaction between 24 hour BP and log UAE but only in DD genotype
{Redon, 2000 #75}	*Angiotensin converting enzyme (ACE)*	Hypertension Caucasian	136	Prospective (Quantitative trait)	ACE I/D	D	Co-dominant	No differences in UAE between genotypes at baseline neither for the slope of UAE during evolution; Significant interaction between the slopes of SBP and UAE only in carriers of DD genotype.
{Pontremoli, 2000 #106}	*Angiotensin converting enzyme (ACE)* *Angiotensinogen (AGT)* *Angiotensin AT1-receptor (AGTR1)*	Italian *(HTN)*	215	Cross-sectional (Quantitative trait)	ACE/I/D AGT/ p.M235T AGTR1/A1166C ACE/ I/D+ AGT/ p.M235T+ AGTR1/A1166C	D T C D+T+C	Dominant None None Dominant	DD+DI genotypes had significant higher levels of ACR and bigger LVMI than II genotype No association with ACR individually No association with ACR individually Subjects carrying the following genotypes (DD/ID en ECA, TT/MT en AGT y AC/CC en AT_1R) had an increase risk of TOD
{Mallamaci, 2000 #100}	*Angiotensin converting enzyme (ACE)*	Hypertension Caucasian	45/343	Retrospective (Case/Control)	ACE/I/D	D	Dominant	D allele was associated with nephroangiosclerosis diagnosed by biopsy
{Chaves, 2001 #103}	*Angiotensin AT1-receptor (AGTR1)*	Spanish (HTN)	183	Cross-sectional (Quantitative trait)	AGTR1/A1166C AGTR1/ C573T	C C	Co-dominant Co-dominant	No association of this SNP with UAE. No association of this SNP with UAE. (After log transformation subjects with the C573C genotype had higher levels of logUAE than the others genotypes)
{Wang, 2001 #116}	*Angiotensin converting enzyme (ACE)* *Aldosterone syntase (CYP11B2)*	Belgian (General population)	159/1295	Cross-sectional	ACE/I/D CYP11B2/-344C/T	D C	None None	No association with mild renal insufficiency (CC≤60ml/min) individually No association with mild renal insufficiency CC≤60ml/min) individually
{Nicod, 2002 #113}	*Angiotensin converting enzyme (ACE)* *Angiotensinogen (AGT)*	Swiss (ESRD)	260/260	Retrospective	ACE/I/D AGT/ p.M235T	D T	None None	No association with ESRD No association with ESRD

Table 3.1. *Resume of candidate gene studies in HTN (II).*

Author, year (ref)	GENE	POPULATION	SAMPLE SIZE (Cases/Controls)	STUDY DESIGN	GENE/POLYMORPHISM	RISK ALLELE	MODEL	Results
RAAS system								
{Marin, 2004 #104}	*Angiotensinogen (AGT)*	Spanish (HTN)	131	Prospective (Quantitative trait)	AGT/ p.M235T AGT/ A-6G	T A	Co-dominant Co-dominant	No association of this polymorphism with changes of UAE along time Patients with the A-6A genotype had lower reduction in UAE along time than the other genotypes
{Fabris, 2005 #101}	*Angiotensin converting enzyme (ACE)* *Angiotensinogen (AGT)* *Angiotensin AT1-receptor (AGTR1)* *Aldosterone syntase (CYP11B2)*	Hypertension Caucasian	86/172	Cross-sectional (Case/Control)	ACE/ I/D AGT/ p.M235T AGTR1/A1166C CYP11B2/-344C/T	D T C C	Co-dominant/Recessive Co-dominant/Recessive Dominant Recessive	Association of genotypes of different polymorphism in RAAS gene with renal failure (Cr>1.5 mg/dl) under different genetic models. Besides the interaction AGT p.T235T+AGTR1 A1166C or CYP11B2 -344C/C+ACE DD was also associated with renal insufficiency
{Pedrinelli, 2006 #115}	*Angiotensin converting enzyme (ACE)*	Italian *(HTN)*	61/177	Cross-sectional (Quantitative trait)	ACE/ I/D	D	None	No association with microalbuminuria
NATRIURETIC PEPTIDE								
{Nannipieri, 2001 #111}	*human Atrial Natriuretic Peptide*	Italian *(Type 1 and 2 diabetes and HTN)*	328/705	Cross-sectional	hANP/ ScaI hANP/708C>T	A^1 T	Allelic test Allelic test	A^1 allele was significantly lower in micro and macroalbuminurics subjects than in normoalbuminurics T allele was significantly lower in microalbuminurics subjects than in normoalbuminurics
{Nannipieri, 2003 #112}	*human Atrial Natriuretic Peptide*	Mexican *(General Population)*	663/625	Cross-sectional	hANP/ ScaI hANP/708C>T	A^1 T	Allelic test Allelic test	A^1 alele was an independent protection factor against microalbuminuria T allele was an independent protection factor against microalbuminuria
ALPHA ADDUCINE+RAAS								
{Wang, 2001 #116}	*Alpha Adducin (ADD)* *Angiotensin converting enzyme (ACE)*	Belgian (General population)	159/1295	Cross-sectional	ADD/ p. *Gly460Trp* ADD/ p. *Gly460Trp*+ ACE/ I/D	Try Try+D	None Dominant	No association with mild renal insufficiency (CC≤60ml/min) individually DD+DI genotypes were associated with a greater prevalence of mild renal insufficiency than I genotype, but only in carriers of the 460Try allele for ADD/ p. *Gly460Trp* polymorphism
{Nicod, 2002 #113}	*Alpha Adducin (ADD)* *Angiotensin converting enzyme (ACE)*	Swiss (ESRD)	260/260	Retrospective	ADD/ p. *Gly460Trp* ADD/ p. *Gly460Trp*+ ACE/I/D	Try Try+ D	None Recessive	No association with ESRD Doble homozygotes for the minor allele in this two SNPs (DD ACE I/D+Try460Try ADD) had a more rapid progression to ESRD since the onset of renal disease than the others genotypes
{Pedrinelli, 2006 #115}	*Alpha Adducin (ADD)* *Angiotensin converting enzyme (ACE)*	Italian *(HTN)*	61/177	Cross-sectional (Quantitative trait)	ADD/ p. *Gly460Trp* ADD/ p. *Gly460Trp*+ ACE/ I/D	Try Gly+D	None Recessive	No association with microalbuminuria Doble homozygotes (DD ACE I/D+Gly460Gly ADD) had a greater prevalence of microalbuminuria than the others genotypes

Table 3.1. *Resume of candidate gene studies in HTN (III).*

Author, year (ref)	GENE	POPULATION	SAMPLE SIZE (Cases/Controls)	STUDY DESIGN	GENE/POLYMORPHISM	RISK ALLELE	MODEL	Results
ADRENERGIC SYSTEM								
(Kobayashi, 2005 #128)	*Adrenomedullin (AM)*	Japanese (HTN)	205/210	Cross-sectional	Microsatellite rs4399321 rs7944706 Microsatellite+ rs4399321+ rs7944706	19 A G 19+A+G	None None None Haplotype	No association with individual polymorphism No association with individual polymorphism No association with individual polymorphism The haplotype including the allele 19 of the microsatellite polymorphism, allele A of rs4399321 and allele G of rs7944706, was associated with microalbuminuria
(Masuo, 2007 #122)	*Adrenergic Receptor β type 2 (ADRB2)*	Japanese (General Population)	219	Prospective (Quantitative trait)	ADRB2/ p.Arg16Gly	Gly	Allele	Subjects with high plasma norepinephrine levels at the beginning, who carry the 16Gly allele, had significantly lower CC after 5 years of follow up and greater reductions in CC over the 5 years period compared to those without the 16Gly allele.
OXIDATIVE STRESS								
(Nagase, 2003 #130)	*endothelial Nitric Oxide Synthase(eNOS)*	Japanese (ESRD)		Cross-sectional	eNOS/Glu298Asp eNOS/ 4a /4b	Asp 4a	Dominant Dominant	The prevalence of ESRD is higher in carriers of the Asp allele than in homozygotes Glu298Glu The prevalence of ESRD is higher in carriers of the 4a allele than homozygotes 4b/4b but only in non diabetics sunjects
(Dell'Omo, 2007 #129)	*endothelial Nitric Oxide Synthase(eNOS)*	Italian (HTN)	235	Cross-sectional	eNOS/Glu298Asp eNOS/c. T786C eNOS/ 4a /4b	Asp C 4a	None None None	No association with microalbuminuria No association with microalbuminuria No association with microalbuminuria
KALLIKREINS								
(Yu, 2002 #135)	*Kallikrein 1 (KLK1)*	African-American (ESRD)	107/85	Cross-sectional	KLK1/Promoter (-130(G)N)	12G	Allele	12G allele was associated with hypertensive ESRD
KIDNEY STRUCTURAL COMPONENTS								
(Freedman, 2009 #138)	non-muscle Myosin Heavy chain 9 gene (MYH9)	African-American	696/948	Cross-sectional	MYH9/ rs4821480 MYH9/ rs5756152 MYH9/ rs12107 MYH9/rs4821480,+rs2032487+ rs4821481+rs3752462 MYH9/ rs7078+ rs12107+ rs735853+ rs5756129	G A G	Recessive Additive Recessive Haplotype Haplotype	Polymorphisms and one haplotype of this gene showed association with ESRD

ACE: Angiotensin converting enzyme; AGT:Angiotensinogen; AT1R: Angiotensin 1 receptor; hANP: Human Atrial natriuretic peptide; ADRBR2: Adrenergic receptor beta 2; eNOS: endothelial nitric oxide synthase; KLK1: Kallikrein 1; MYH9: non-muscle myosin heavy chain 9 gene.

3.4.2. Linkage studies

Several linkage studies have been published to date. The HyperGen study found a heritability rate for the creatinine clearance of 0.17 and 0.18 in African-Americans and in Caucasian-American families of essential hypertension, respectively [140]. This study found a significant logarithmic of odds score (LOD) values on chromosome 3 (specially in African-American subjects (LOD=3.61, to 214.6 centimorgans, 3q27) according to the scale of Lander and Kruglyak in an adjusted model [141]. There are two candidate genes in this region of chromosome 3, the 3-hydroxyl-coA dehydrogenase gene and the apolipoprotein D gene. The former can facilitate the accumulation of long chain fatty acid in the tubular cells of the kidney, and the second might produce atherosclerosis in renal vessels[140]. Using the albumin/creatinine ratio within the same HyperGen population, the previous results were not be replicated by Freedman et al[142]. In the Freedman study, the albumin/creatinine ratio heritability after adjusting for confounding factors was 0.49, and the susceptibility loci were located on chromosomes 12 and 19 (maximum LOD=2.40 , to 9cM on chromosome 19)[142] . The low density lipoprotein (LDL) receptor gene is located in this chromosomal region (19p13). Therefore, those genes which are linked to atherosclerosis seem to play a role in the renal damage associated with HTN. This can be due to the endothelial damage and atherosclerosis of the small and large vessels[142].

It is noteworthy to mention some efforts to identify in humans the major loci related with albuminuria and renal failure in the Fawn Hooded Rats[143]. These genes, called Rf-1 and Rf-2, are located on chromosome 1 which corresponds to the human chromosome 10. One linkage study in this chromosome found a significant linkage peak in 10p for the ESRD in African-Americans, but failed to find an association in the human homologue of Rf-1[144]. The same group, in 356 African-American sib-pairs concordant for ESRD, including a large number of sib-pairs with hypertensive ESRD, found significant evidence for linkage for one marker close to the Rf-1 gene (LOD score 3.4, p=0.0004)[145]. The association of ESRD with the marker *D10S677* was present in the whole group, in type 2 diabetic ESRD and in the non-diabetic ESRD group[145]. The study was also able to replicate the previous linkage peak on chromosome 10p in the total sample and in the non-diabetic group, but not in the type 2 diabetic ESRD or the late onset ESRD[145]. The linkage peak in the long arm of chromosome 10 was also replicated in another study in 49 Utah Caucasian-Americans selected by premature cardiovascular disease[146]. In this case the association was with creatinine clearance in three visits during 10 years of follow-up (LOD score for *D10S677*, 1.4 in the first visit)[146]. The best peak of association for creatinine clearance was in examination 3 (*D10S2470*, LOD score 2.09)[146]. Interestingly, this peak is close to the one found in examination 1 and in the previously described African-Americans[145, 146]. The heritability for creatinine clearance increased from 0.33 in the first to 0.53 in the third examination. They also found a heritability of 0.20 for the change in creatinine clearance between the first two visits and 0.40 for the change in creatinine

clearance between the first and the last measurement[146]. These studies suggest that there is a susceptibility loci for renal failure on chromosome 10 at least in hypertensives with RI [144-146]. These loci, however, seem to be different in regards to the etiology of renal failure.

In the general population, one linkage study within the Framingham Heart Study suggests a high heritability component for serum creatinine, glomerular filtrate rate (GFR) and creatinine clearance (0.29, 0.33 and 0.46 for each phenotype, respectively)[147] . The association loci for these traits were located in different chromosomes: 3q,4q and 11p (maximum LOD=2.28 , 176 cM in the long arm of chromosome 4 for serum creatinine)[147] . Within the associated chromosomal regions, there are several candidate genes, such as: activated factor for the TNF receptor gene, interleukin 8 gene (IL-8), albumin gene and vascular endothelial growth factor (VEGF) gene[147] . The authors hypothesize that the interaction between those genes would produce glomeruli inflammation and renal damage[147]. In a later work with the same population of Framingham, the heritability for microalbuminuria was 0.16 and a suggestive LOD score for the association was found on chromosome 8 (LOD=2.22 , to 135 cM)[148]. This result was replicated in a subanalysis in hypertensive families[148]. The hialuron synthase gene falls in that region of chromosome 8. This gene is expressed in the tubular cells of the kidney and has been related with acute and chronic renal cortex scarring[149].

The Strong Heart Family Study[150] included a large cohort of American-Indian individuals enriched by cardiovascular risk factors. Within this cohort, a linkage study for GFR revealed a significant QTL on chromosome 12p (LOD 3.5 and 4.6 after removing those individuals after hypertensive therapy)[150]. Interestingly, a similar peak on chromosome 12 had been previously described in African-American type 2 diabetics[151]. Although not the objective of the study, the authors also analyzed the linkage for ACR, finding no evidence for linkage. It is noteworthy that within that region there lies a possible candidate gene which codifies the glomerular epithelial protein 1 (GLEPP1)[152].

A resume of the main linkage studies for renal traits in essential hypertension is shown in table 3.2 (I,II).

Table 3.2. *Resume of linkage studies in HTN (I).*

Study	Population	Sample size(N/Composition)	Study Design	Phenotype	Heritability	Chromosomal region
{Yu, 1999 #144}	African-American (HTN+DM2+ other aetiologies of ESRD)	258 (129 sibling pairs)	Cross-sectional	ESRD		10p15.3
{DeWan, 2001 #140}	Afro-American(AA) European-American(EA) Both (Hypertensive siblings)	466 (215 sibships) 651 (265 sibships) 1117 (480 sibships)	Cross-sectional	CC	0.17 (AA) 0.18 (EA)	3q27
{Freedman, 2002 #145}	African-American (HTN+DM2+ other aethiologies of ESRD)	668 (356 sibpairs, 296 families, 157concordant for type 2 diabetics ESRD and 199 concordant for non Diabetics ESRD)	Cross-sectional	ESRD		10q23.33
{Hunt, 2002 #146}	European-American (General population - Pedigrees were ascertained for cardiovascular events or HTN)	1360 (49 pedigrees)- Examination 1 1196 (49 pedigrees)- Examination 2 718 (49 pedigrees) -Examination 3	Cross-sectional Follow-up	CC Changes in CC along 10 years of follow-up	0.33- Examination 1 0.36- Examination 2 0.53- Examination 3 0.20 (between E1 and E2) 0.49 (between E1 and E3)	10q23.33 10q23.33
{Freedman, 2003 #142}	Afro-American European-American Both (Hypertensive families)	834 893 1727 (1164 sibling pairs, 22 parent-offspring pairs, 61avuncular pairs, 2 half-sibling pairs, 4 first cousins, 5 identical sibling pairs, and 37 unrelated pairs. The mean family size with ACR data were 2.03 members)	Cross-sectional	UACR	0.49	19p13 12q21
{Fox, 2004 #147}	European-American (mainly) (General population)	1224 (330 pedigrees and 546 sibships)	Cross-sectional	Serum creatinine GFR CC	0.29 0.33 0.46	4q32.3/11p13 4q13.2 3q26.31/4q13.2
{Fox, 2005 #148}	European-American (mainly) (General population)	1055 (330 families) 676 (in the subset enriched for hypertension)	Cross-sectional	UACR	0.16 0.20 (in the hypertension enriched subgroup)	8q24.13 8q24.13(in the hypertension enriched subgroup)
{Mottl, 2008 #150}	American-Indian (General population enriched for hypertension (30%)	3665 (90 extended families originating from 13 separate American Indian tribes)	Cross-sectional	eGFR	0.33 (Whole sample) 0.33 (Arizona) 0.33 (Dakota) 0.33 (Oklahoma)	12p12.2 1p36.31 2q33.3 (Only Dakotas) 9q34.2 (Only Dakotas) 12p12.2 (Excluding subjects der hypertensive treatment)

Table 3.2. *Resume of linkage studies in HTN (II).*

Study	Chromosomal region	Main associated markers	LOD score	Results	Possible candidate genes
{Yu, 1999 #144}	10p15.3	D10S1435 D10S249	1.77 (Whole sample) 1.52 (Non diabetic ESRD) 1.53 (Early onset ESRD)	Association of two markers in 10p15.3 with ESRD in non diabetic ESRD. There was not association with the human homologous region of Rf-1.	
{DeWan, 2001 #140}	3q27	D3S2398 D3S2418	3.61	Best evidence of linkage for CC in a fully Adjusted model in African-Americans. Linkage regions are different between African-American and whites	Hydroxyl-coA dehidrogenase (3q27.2) Apolipoprotein D gene (3q29)
{Freedman, 2002 #145}	10q23.33	D10S677 D10S1435	3.4 (Whole sample) 2.35 (Non diabetic ESRD) 2.08 (Type 2 diabetic ESRD 2.82 (Early onset ESRD)	Association of one marker, adjacent to the homologous Rf-1, with all causes of ESRD, type 2 diabetes ESRD and non diabetes ESRD. They confirmed the previously associated Marker in 10p in non diabetic ESRD in AA.	Human homologue Rf-1 gene (10q24-26)
{Hunt, 2002 #146}	10q23.33 10q23.33	D10S677 D10S1230 D10S2470 D10S1230 D10S2327	1.42-Examination1 1.94-Examination 2 2.09-Examination3 1.68 (between E1 and E2) 1.05 (between E1 and E3)	Replication in European-American of the linkage peak previously associated with renal damage In hypertensive rats and ESRD in Afro-Americans	Human homologue Rf-1 gene (10q24-26
{Freedman, 2003 #142}	19p13 12q21	D19S591 PAH	2.40 1.75	Evidence of linkage to UACR in chromosomes 19 and 12 in hypertensive families.	LDL receptor (19p13.2)
{Fox, 2004 #147}	4q32.3 /11p13 4q13.2 3q26.31/4q13.2	D4S2368/ D11S1392 D4S2367 D3S2427/ D4S2367	2.28 and 2.19, respectively 2.19 1.91 and 1.33	Evidence of linkage between renal traits and chromosomes 3,4 and 11	IL-8 (CXC chemokine family) (4q13.3) TRAF6 (11p12) Albumin gene (4q13.3) VEGF C (4q34.3)
{Fox, 2005 #148}	8q24.13 8q24.13(in the hypertension enriched subgroup)	D8S1179 D8S1179	2.22 2.11(in the hypertension enriched subgroup)	Evidence of linkage between UACR and chromosome 8 in the whole sample and in the subset enriched for hypertension	HAS2 (8q24.13)
{Mottl, 2008 #150}	12p12.2 1p36.31 2q33.3 (Only Dakotas) 9q34.2 (Only Dakotas) 12p12.2 (Excluding subjects der hypertensive treatment)	D12S310 D1S214 D2S325 D9S164 D12S310 D12S31	3.5 †/2.1†† 2.3 †/2.0†† 1.8 †/0.5 †† (Only Dakotas) 2.4 † / 1.7†† (Only Dakotas) 4.6†/2.8††	Evidence of linkage between eGFR and chromosome 12p. There were not interaction between genes and hypertension, neither for diabetes or obesity.	PTPRO (candidate gene for GLEPP-1)

CC: Creatinine clearance; UACR: Urinary albumin creatinine ratio; GFR: glomerular filtrate rate; TRAF6: tumor necrosis factor receptor–activating factor; IL-8: Interleukin 8 gene; VEGF-C: Vascular endothelial growth factor C gene; UACR: Urine albumin/creatinine ratio; HAS2: hyaluron synthase 2; * p-value for the family based association test (FBAT); ADAM23: ADAM metallopeptidase domain 23: PCDH9: protocadherin 9; eNOS: endothelial nitric oxide synthase; †Model 1 was adjusted for age, sex, age squared and age-sex interactions; ††Model 2 was additionally adjusted for diabetic status, body mass index, systolic blood pressure, diastolic blood pressure, high density lipoprotein cholesterol, low density lipoprotein cholesterol, triglycerides and smoking status; PTPR0: protein-tyrosine phosphatase receptor type-O ; GLEPP-1: glomerular epithelial protein 1.

3.4.3. Whole genome association studies and combined strategies

Urinary albumin excretion in hypertension was assessed within the British Genetics of Hypertension (BRIGHT) study[153]. EUA was measured in 24-hour urine samples. More than a half million of SNPs were evaluated in order to assess the association with blood and urine biomarkers. Between them, two SNPs, on chromosomes 3 and 12, showed an association with urine albumin, but none of them was selected for replication in others populations[153].

In general populations, there is one example of a family-based association study within the "NHLBI´s Framingham Heart Study". The authors assessed several markers of the renal function, including UAE. Two statistical analyses, the family-based association test (FBAT) and the generalized estimating equations (GEE), were performed to evaluate the strength of the association with the traits[154]. Several SNPs in different chromosomes were associated with UAE[154]. One of the associated SNPs, *rs2113379*, is located in an intronic region within the ADAM23 which belongs to the metalloprotease gene family. They also identified another possible candidate gene, PCDH9, which belongs to the cadherin gene family[154]. The main weakness of this study, due to the variability and circadian pattern of the UAE, was that it used an isolated urine sample instead of 24-hour urine sample to determine it[154]. An attempt was made to replicate the SNPs most associated with renal damage, within the participants of the Atherosclerosis in Communities study (ARIC study)[155]. Unfortunately none of the SNPs associated with the UAE were selected for replication. In this study, one intronic SNP within the methenyltetrahydrofolate synthetase (MTHFS) seems to be associated with CKD, and the allele *C* seems to increase the risk of kidney disease progression in Caucasian-Americans, but not in an African-American population[155]. Again these results must be cautiously considered because the replication was only for CKD, but not for the estimated GFR or serum cystatin C and in only one of the successive visits.

As an example of combined strategies, there is one running Italian study which is evaluating the genetic influence in the response to losartan treatment in a cohort of untreated hypertensive patients[156]. Several variants in different genes previously described are being evaluated, including genes of the RAAS, adrenergic system, kinin-kallikrein, sodium handling and drug metabolism. Half of the population is also being used to perform a GWAS, with more then 1 million SNPs. Although it is not the main objective of the study, the genetic association with intermediate phenotypes will be tested. Among the intermediate phenotypes to be analyzed is microalbuminuria based on several 24-hour urine collections during follow up. Perhaps this study can discover new variants in different genes or clarify the role of the previously described variants.

A resume of the main genome wide association studies for renal traits in essential hypertension is shown in table 3.3.

Table 3.3 *Resume of genome wide association studies in HTN .*

Study	Population	Sample size (N)	Study Design/Platform	Phenotype	Chromosomal region	Main associated markers	p-values	Results	Possible candidate genes
{Hwang, 2007 #154}	*Framinghan heart study/ White Americans/ European descent (General Population)*	1345	Population based/ Cross-sectional (Different visits)/ Affymetrix GeneChip Human Mapping 100k	UACR	*10p14*	*rs1243400*	$4.8*10^{-08}$/0.036	Several SNPs were significantly Associated with UACR	
					10p14	*rs827640*	$1.5*10^{-08}$ /0.047		
					12p11.21	*rs7315682*	$2.1*10^{-08}$/0.28		FGD4 (12p11.21)
					1q42.2	*rs1543468*	$2.5*10^{-08}$/0.253		SLC35F3 (1q42.2)
					4q28.3	*rs723464*	$2.7*10^{-08}$/1.0*10-05		
					5q35.1	*rs33855*			
					12q23.3	*rs2374688*	$6.2*10^{-08}$/0.192		BTBD11(12q23.3)
					15q22.2	*rs10519012*	$9.1*10^{-08}$/0.087		
					11p12	*rs10501264*	$1.0*10^{-07}$/0.088		
					12q24.13	*rs10492025*	$1.1*10^{-07}$/7.2*10-04		RPH3A (12q24.13)
					7q32.1	*rs2056694*			
					19p13.11	*rs8113386*	$1.1*10^{-07}$/0.41		KLF2 (19p13.11)
					11q23.2	rs1712790	$1.1*10^{-07}$/0.1		FAM55B (11q23.2)
					21q21.3	rs9305354	$1.1*10^{-07}$/0.053		
					2q33.3	rs2113379	0.014 /$1.9*10^{-08}$		ADAM23 (2q33.3)
					1p36.31	rs278021	0.013/ $8.4*10^{-08}$		DNAJC11 (1p36.31)
					9p13.3	rs1856190	0.003/ $1.4*10^{-08}$		
					6q15	rs10485409	0.958 /$2.9*10^{-08}$		
					1q41	rs2785980	0.127/ $3.0*10^{-08}$		
					3q28	rs837678	0.147 /$3.1*10^{-08}$		LEPREL1(3q28)
					13q32.3	rs2761171	$4.8*10^{-07}$/ $3.7*10^{-08}$		CLYBL (13q32.3)
					11q23.2	rs10502192	0.012 /$3.8*10^{-08}$		FAM55B (11q23.2)
							0.078/ $4.1*10^{-08}$		
							0.051/ $4.9*10^{-08}$		DNAH7(2q32.3)
				sGFR	2q32.3	rs6434804	$2.4*10^{-08}$/0.003	Several SNPs were significantly Associated with eGFR	PRKCH (14q23.1)
					18q12.3	rs2885618	$4.8*10^{-08}$/$1.0*10^{-07}$		PCNT2 (21q22.3)
					14q23.1	rs10483741	$6.3*10^{-08}$/ 0.298		
					21q22.3	rs2839235	$1.6*10^{-08}$/0.055		PABPC3 (13q12.13)
					13q14.3	rs3095160	$3.8*10^{-08}$/0.002		
					13q12.13	rs10507344	$4.1*10^{-08}$/0.017		CD48 (1q23.3)
					5q33.3	rs890945	$4.7*10^{-08}$/0.036		
					1q23.3	rs10489639	$4.9*10^{-08}$/0.194		
									EPAS1 (2p21)
				Cys C	18q22.1	rs563754	$4.7*10^{-08}$/ 0.002	Several SNPs were significantly Associated with Cys C	
					2p21	rs2121267	$6.6*10^{-08}$/ 0.002		
					11q14.1	rs1400306	$7.2*10^{-08}$/0.061		
					8q12.1	rs10504244	$8.3*10^{-08}$/0.005		CST9L|CST9|CST3 (20p11.21
					6q14.1	rs1931619	$1.0*10^{-07}$'0.05		CST9|CST3|CST4 (20p11.21)
					20p11.21	rs1158167	$8.5*10^{-07}$/0.006		
					20p11.21	rs2145231	$1.1*10^{-08}$/0.011		
{Wallace, 2008 #153}	Bright study/ Whites- European origin (HTN)	1144	HTN/Cross-sectional (Affymetrix GeneChip Human Mapping 500K)	UACR	12q23.3	rs11111839	2.51 3 10^{-7}	Two markers were significantly Associated with urinary albumin	XR_015316.1* (12q23.3)
					3p24.3	rs17006217	4.52 3 10^{-7}		
{Kottgen, 2008 #155}	ARIC study/ European-American and African-American *(General Population)*	15747 (11,447 Caucasian and 4253 African-American)	Population based/ Cross-sectional/Most associated SNPs in the FHS 100K {Hwang, 2007 #125}	CKD	15q25.1 (EA)	rs6495446	0.001	One intronic SNP within the methenyltetrahydrofolate synthetase (MTHFS) seem to be associated with CKD and the allele C seem to increase the risk of kidney disease progression in whites but not in black population	MTHFS (15q25.1)

FGD4: FYVE, RhoGEF and PH domain containing 4; SLC35F3: solute carrier family 35, member F3; BTBD11:BTB (POZ) domain containing 11;RPH3A: Rabphilin-3A (Exophilin-1); KLF2: Krueppel-like factor 2; FAM55B: family with sequence similarity 55, member B;ADAM23: ADAM metallopeptidase domain 23; DNAJC11: DnaJ (Hsp40) homolog, subfamily C, member 11;LEPREL1:Prolyl 3-hydroxylase 2 Precursor; CLYBL :citrate lyase beta like; DNAH7: Dynein heavy chain 7, axonemal ;PRKCH: Protein kinase C eta type; PCNT2: Pericentrin (Pericentrin B)(Kendrin) ; PABPC3: Polyadenylate-binding protein 3 ;CD48: antigen Precursor (B-lymphocyte activation marker BLAST-1)(BCM1 surface antigen); EPAS1: Endothelial PAS domain-containing protein 1; CST9L: Cystatin-9-like Precursor; CST9: Cystatin-9 Precursor (Cystatin-like molecule);MTHFS: methenyltetrahydrofolate synthetase

3.4.4. Summary of genetics of microalbuminuria in HTN and general population

Despite the great efforts that have been made in the field, knowledge about the major genetic variants causing the susceptibility to develop renal damage in HTN is scarce.

In general, candidate gene strategy has led to conflicting results. Although there are associations with some variants which have been replicated, the majority was not or showed an association only in certain subgroups of patients. The RAAS system is probably the principal system involved not only in the HTN-induced renal damage association, but also in the risk for HTN itself. One should take into account, however, that this system is the most studied one, and that there is a bias to publish only positive results. The bias for positive results and the heterogeneity of the different studies, put in doubt the results of the meta-analysis. The greatest degree of evidence is for the *D* allele of the *I/D* polymorphism in the ECA gene. This allele has been related to the blood levels of ECA[157] which increases the possibility of a functional role for this variant. Although there are some conflicting results, cross-sectional and follow-up studies give credit to its role as a susceptible variant for several renal traits in HTN. Supporting the potential role of the *D* allele in renal damage are the results obtained in type 1 diabetes. A large case control study in three different European type 1 diabetic populations demonstrated that the *D* allele alone or in haplotypes with others was associated with the risk to develop albuminuria[158]. The evidence for other variants of the RAAS is less clear, although there are several studies which support the interaction between different genetic variants on the system[101, 106].

The appearance of new candidate genes, such as the non-muscle myosin heavy chain 9 gene (MYH9) is remarkable. This gene is a strong candidate gene for renal failure associated with hypertension in the African-American population[138, 139]. We still do not know what the exact contribution of these genes to the renal damage is nor if their influence varies among populations.

Linkage studies have given credit to some of the previously described candidate genes and have also identified others. The high heritability for different renal traits in this kind of study supports the potential role of genetic factors in interaction with environmental factors[142, 147, 148]. This heritability seems to be higher for the quantitative trait albumin/creatinine ratio than the heritability for qualitative microalbuminuria[142, 148].

Some of the linkage peaks deserve special attention. For example, the linkage peak on chromosome 3q for creatinine clearance which was found in both African-Americans within the HyperGen study as well as in Caucasian-Americans of European descent within the Framingham Heart study[140, 147] contains several interesting genes such as Hydroxyl-CoA-dehydrogenase and apolipoprotein D gene. Another linkage peak for GFR on chromosome 12p, which was found in both Native Americans within the Strong Heart family Study, as well as in African-American type 2 diabetics[150, 151], contains the gene protein tyrosine phosphatase receptor type O (PTPR0), a gene which codifies an epithelial protein expressed in the glomeruli[152]. Likewise, the human homologue

region for the Rf-1 gene in rats on chromosome 10, is another potential loci for renal failure in hypertension according to several linkage studies[144-146]. It is noteworthy that some of the linkage peaks contain genes of lipid metabolism[140, 142] which are thought to be related to the development of atherosclerosis accelerated by HTN [142].

The genome wide association studies (GWAS) have also helped to identify new candidate genes, such as the metalloprotease, ADAM23, and the methenyltetrahydrofolate synthetase (MTHFS) gene[154, 155]. The association of variants in MTHFS with CKD was replicated, although with certain limitations and, therefore, further studies are needed to assess its possible role.

Over the next few years, there will probably be an increasing number of GWAS related to microalbuminuria in hypertension. As far as we know, no large studies of this class have been made with microalbuminuria as the main endpoint.

In summary, the genetic studies for albumin excretion and renal damage have identified several variants in candidate genes and different regions of the genome which may influence the appearance of HTN-induced organ damage. These results have to be taken into cautious consideration because most of them have not been clearly replicated or the results have been controversial. Well-designed, large-scale future GWAS and replication studies with large cohorts can help to clarify the genetic bases of renal damage in HTN.

3.5. Genetic bases of microalbuminuria and related traits in type 1 and type 2 diabetes

3.5.1. Overview

Microalbuminuria also increases both morbidity and mortality in type 1 and type 2 diabetes [3-11, 159]. For this reason it is also important to know which factors lead to its development in diabetic patients. It is clear that the grade of glycemic control and the time of evolution of the disease are between the main factors for the development of microalbuminuria in DM[160, 161]. These factors usually coexist with high blood pressure levels and insulin resistance which have been related with renal damage especially in non diabetics subjects[36-39] . In contrast to retinopathy which appears in almost all the subjects with more than 20 years of evolution of DM, renal damage only appears in a third of patients [162-167]. That's means that there must be another factors, mainly genetics, which contribute to the development of renal damage in type 1 and type 2 diabetes[165]. This genetic susceptibility, in combination with the other factors, may ease the development of microalbuminuria and renal damage in diabetic patients. Although the genetics of microalbuminuria in DM have been widely explored in the last few years, the information in the majority of the cases is confusing.

Epidemiological and family studies strongly support the genetic contribution for microalbuminuria and renal damage in DM. In this way, it has been demonstrated that relatives of patients with DM2 are prone to develop microalbuminuria even if they were not diabetics themselves and this microalbuminuria can finally drive to the end stage renal disease (ESRD)[76, 77]. Also the ESRD itself have a strong family clustering in diabetes and HTN[77, 83]. The genetic susceptibility for microalbuminuria is also supported by the high heritability for the ACR, ranging from 0.30 to 0.44, in families enriched for subjects with type 2 diabetes[77, 168-170], and also for the differences in renal disease between ethnics[171]. For example, Afro-Americans or Hispano-Americans[78-80] are prone to develop albuminuria and renal disease. It seems that the differences between ethnics are more due to different genetic backgrounds than related to environmental factors such as different lifestyle or inequalities in the availability of the treatments [81].

Although genetic factors play an important role for the development of renal damage in DM, what are the main genomic variants involved it is not as clear. The next pages resume some of the studies which have tried to unravel the genetic susceptibility variants for diabetes associated nephropathy.

3.5.2. Candidate gene studies

Some of the systems or physiological pathways which have been investigated are: renin-angiotensin-aldosterone system (RAAS), fibrinolysis, oxidative stress, lipid metabolism, growth factors and glucose metabolism.

A resume of some of the candidate gene studies for renal traits in type 1 and type 2 diabetes is shown in table 3.4 (I-IV).

3.5.2.1. RAAS genes

The *I/D* polymorphism has been widely explored in diabetes patients. Although there are some positive studies for the association of the *D* allele with DN, not all the studies support this association[172, 173] . Besides the meta-analysis[174, 175] to analyze the relationship of this polymorphism of ACE with diabetes nephropathy are inconclusive due to the heterogeneity of the studies. However, one large case control study in three different European populations has given credit to the role of the *D* allele, alone or in haplotypes with others alleles, in the development of albuminuria in type 1 diabetes[158]. Another study in this case in type 2 diabetes with advanced nephropathy failed to find positive association with individual variants of ACE gene but not with haplotypes[176].

The *TT* genotype of the polymorphism *p.M235T*, in the AGT gene, as well, have been related to the risk of diabetic nephropathy individually or in association with other polymorphisms in the RAAS system genes[177-179]. One consideration to take into account is that this association seems to be strongly influenced by gender[177-179].

3.5.2.2. Fibrinolysis

The activated plasminogen inhibitor (PAI-1) because of its functions in the intravascular fibrinolysis, tissue healing and remodelling, might also be involved in the glomerular lesions[180]. One common single-base-pair guanine *I/D* polymorphism in the promoter region of the PAI-1 gen (PAI-1 *4G/5G I/D*) could regulate the blood levels of PAI-1[181, 182]. One study carried on Caucasian population aimed to detect association between this PAI polymorphism and renal impairment in type 1 diabetics[180]. None differences in genotypes were found between people with or without renal damage[180] . However subjects with microalbuminuria had a trend to present higher blood PAI-1 levels than those without it[180]. Similar non conclusive results were obtained in an Austrian population of subjects with type 2 diabetes regarding to the UAE[183]. Nevertheless this study found a positive association with proliferative diabetic retinopathy[183]. Genotype *4G/4G* might be more frequent in subjects with albuminuria although that was only a trend without statistical significance[183] .

3.5.2.3. Oxidative stress

The *p. V16A* polymorphism in the Manganese Superoxide dismutase gene (MnSOD) has been widely explored in diabetes. This polymorphism was associated with different stages of UAE in a Korean population of subjects with type 2 diabetes[184]. The *A* allele seems to be protective against the development of microalbuminuria[184]. The endothelial nitric oxide synthase (eNOS) gene has been also associated with renal impairment and microalbuminuria in diabetics. Both the allele *C* of the *786 T>C* polymorphism and the deletion allele of the *I/D* polymorphism of eNOS, increase the risk of advanced DN in Caucasian subjects with type 1 diabetes[185]. Mainly in Asian diabetic populations, other polymorphisms of this gene such as *tamden repeats* and the missense mutation (*p. Glu298Asp*) in exon 7, were related to the risk of ESRD[130]. There is not agreement for the association between all the studies. For example, in a British population of type 1 diabetics, Rippin et all[186] were not able to find association with nephropathy.

Other genes implicated are the paraoxonase 1(PON-1) and 2(PON-2) which codify enzymes involved in the protection against the oxidative stress. Unsurprisingly, several of the studied polymorphisms (*p.Gln192Arg* in PON1, *p.Ala148Gly* and *Cys311Ser* in PON2) did not show association with nephropathy in a Russian population of type 1 diabetics[187].

3.5.2.4. Lipid metabolism

It is noteworthy that the previous authors[187], who failed to find association with oxidative stress genes, in the same population of type 1 diabetics, did find association with polymorphisms in another kind of genes belonging to the lipid metabolism. Special attention deserves the allele *epsilon 2* of one multiallelic polymorphism which codifies three isoforms (*epsilon 2/epsilon 3/epsilon 4*) in the Apolipoprotein E (apo-E) gene. This isoform, *ε2*, has been related with DN in several papers[188-192]. Besides this polymorphism showed to be independently associated with microalbuminuria in both type 1 and type 2 diabetics[193]. However this result is not supported by all the studies. One Russian study found relationship between the allele *ε3* and the risk of DN and albuminuria (≥300 mg/day)[194] . In this study other allele, *D*, of the *I/D* polymorphism in Apolipoprotein B (apo-B) gene showed to be protective against the presence of DN and albuminuria[194]. One case-control study in Chinese population of type 1 diabetics did not find association between the *S447X* in the Lipoprotein Lipase (LPL) gene, the *455T>C* SNP in the apoC3 gene and the *ε2,ε3*, and *ε4* isoforms in the ApoE and the risk of DN, individually[195]. Nevertheless some combination of genotypes for the previous SNPs may have a protective role against DN[195] .

3.5.2.5. Growth factors

Between them, transforming growth factor beta (TGF-β) gene is one of the most important ones because of its profibrogenic properties. This gene is involved in the regeneration of the tissue and fibrosis which occurs after an injury in several organs including the kidney[196].

Animal models of DN showed an increase in the messenger ribonucleic acid (RNA) TGF- β1 in the glomeruli, tubule, and interstitium[197]. Also in animals there is an upregulation in TGF- β receptors in those with glomerulosclerosis[198, 199]. Taking into account these facts, several polymorphisms in this gene TGF- β or in the receptors genes (TGFβR1 and TGFβR2) tried to be linked with DN and microalbuminuria with inconclusive results. Patel et all[200] found a significant association between the *Leu10* allele of the SNP *p.Leu10Pro* (*rs1982073*) of the TGF-β1 gene and the risk of DN. Interestingly this study included a large number of subjects with micro or macroalbuminuria (35.7%)[200]. However not all the studies support this association[201, 202].

3.5.2.6. Glucose metabolism

One of these genes codify the aldolase reductase (ADLR1) which is the key enzyme in the poliols pathway that reduces the nicotiamide-adenin dinucleotide phosphate (NADPH) depends on glucose to sorbitol[203]. It is remarkable that this way is only activated in presence of hyperglucemia [203]. Two are the main factors contributing to the organ damage associated with hyperglucemia: the accumulation of sorbitol due to its osmotic effects and the decrease in the NADPH levels affecting the oxidative-reduction status of the cell[203, 204] . Neamat'Allah et all[205], in several populations of type 1 and type 2 diabetics, found an association between the allele *T* of the *106C>T* SNP and nephropathy in 3 out of the 4 studied populations. These authors also made a meta-analysis for another previously associated polymorphism in a microsatellite sequence (*(CA) n*)[206, 207]. This meta-analysis showed no association in type 2 diabetics whereas in type 1 diabetics the association is doubtful [205]. Another gene involve in the glucose metabolism codify the receptor of advanced glycation end products "RAGE". The glycation products are responsible for many adverse effects including the vascular damage[208]. It has been demonstrated in animal models that by blocking the receptor for the glycation products we can prevent the development of atherosclerosis[209, 210]. Moreover there is an over-expression of the receptor in animals with vascular disease[211]. Two polymorphism in the promoter (*-429T>C*, and *-374T>A)* and one missense mutation (*p.Gly82Ser*) have been the most studied ones. The relationship between these polymorphisms and renal damage was investigated in a Finnish population with type 1 diabetics[212]. The prevalence of microalbuminuria was significant less for the genotype *AA* of the *-374T>A* polymorphism but only in the group with poor glycemic control (HbA1c > 9.5)[212]. The authors of this study suggest an interaction between the genetic variants and the environmental

factors[212]. Several variants in different "RAGE" genes were evaluated in Caucasians type 1 diabetics subjects[213] but none of them were clearly associated (only one variant, *C-1152A*, showed a weak association)[213].

The ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1) gene has been associated with insulin resistance and it is expressed in the kidney tubules [214, 215], therefore is a strong candidate gene for DN. This gene was related with DN in type 1 and type 2 diabetes, but it is not clear if this association is due to the diabetes status itself or to the presence of DN[216-218]. In favour of the association with DN, a recently published large collaborative study in type 2 diabetes, showed association between the allele *121Q* of the *K121Q* polymorphism of the ENPP1 gene and a decrease in the GFR at least in two of the three studied populations and in the whole sample[219].

Table 3.4. *Resume of candidate gene studies in DN (I).*

Author, year (ref)	GENE	POPULATION	SAMPLE SIZE (Cases/Controls)	STUDY DESIGN	GENE/POLYMORPHISM	RISK ALLELE	MODEL	Results
RAAS system								
{Fogarty, 1996 #178}	*Angiotensinogen*	DM1 Irish	95/100	Cross-sectional Case-Control	M235T	T	Recessive	TT genotype was associated with an increase of risk of DN (OR 2.7 (1.04-7.52).
{Marre, 1997 #179}	*Angiotensin converting enzyme (ACE)* *Angiotensinogen* *Angiotensin II type 1 receptor (AT1R)*	DM1 French and Belgian (mainly Caucasians)	337/157	Cross-sectional Case-Control	I/D M235T T174M T573C A1166C	D T M C C	Dominant - - - -	II genotype was protective against the risk of DN. There was not association for SNPs in the angiotensinogen gene individually, but there was an interaction between M235T and I/D polymorphism. T allele of the M235 T of angiotensinogen increase the risk of DN but only in carriers of the D allele of the I/D polymorphism of ACE gene. There were nor association for the polymorphism of the AT1R gene and DN
{Fujisawa, 1998 #172}	*Angiotensin converting enzyme (ACE)*	DM1 DM2 Heterogeneous, mainly Asian and Caucasian populations	2495/2278	Meta-analysis 18 Case/Control studies	ACE/ I/D	D	Dominant	ID and DD genotypes had significantly higher risk of DN than II genotype (OR 1.32 (1.15-1.51) both in DM1 and DM2 and also in Asian and Caucasian population when analyzed separately.
{Rogus, 1998 #177}	*Angiotensinogen*	DM1 White Americans	148 case families/62 control families	Cross-sectional FBAT	M235T	T	FBAT	There was not evidence of association between the T allele of M235T and DN. However there were a preferential transmission of this allele T in certain subgroups: ESRD and in males.
{Kunz, 1998 #175}	*Angiotensin converting enzyme (ACE)*	DM1 DM2 Heterogeneous, mainly Asian and Caucasian populations	5336	Meta-analysis 19 Case/Control studies	ACE/ I/D	D	Dominant	ID and DD genotypes had significantly higher risk of DN than II genotype (OR 1.88 (1.42-2.85) but only in Asian populations with DM2. There were not association in Caucasians with type 1 or type 2 diabetes.
{Ng, 2006 #176}	*Angiotensin converting enzyme (ACE)*	DM2 Caucasian	291/167	Cross-sectional Case-Control	A-5466C T-3892C I/D A-5466C+T-3892C+ID	A T D ATD	- Haplotype	There were not association for individual SNPs with advance nephropathy. The haplotype, ATD, containing the D allele of the I/D polymorphism of ACE gene increased significantly the risk of advanced nephropathy

Table 3.4. *Resume of candidate gene studies in DN (II).*

Author, year (ref)	GENE	POPULATION	SAMPLE SIZE (Cases/Controls)	STUDY DESIGN	GENE/POLYMORPHISM	RISK ALLELE	MODEL	Results
RAAS system								
{Hadjadj, 2007 #158}	*Angiotensin converting enzyme (ACE)*	DM1 Three European populations	1057/1127	Cross-sectional Case-Control	ACE/ I/D ACE / rs1800764 ACE/rs4311 ACE/rs4366 ACE/rs12448792	D C T G G	Allelic	D allele was associated with an increase of risk of DN (OR 1.13 (1.02-1.23). All of these allele seem to increase the risk of DN in type 1 diabetic patients.
			244 case families/288 control families	FBAT	rs1800764+ rs4311+ I/D +rs4366+ rs12449782	-DGG	Haplotype	Haplotypes containing the D allele of the I/D polymorphism, And the G allele of the rs4366 and rs12448793 increase the risk of DN There were not association with the D allele of ACE in the family study.
Fibrinolysis								
{Tarnow, 2000 #180}	*PAI-I*	DM1 Caucasians	198/192	Cross-sectional Case-Control	4G/5G	4G	-	There were not association between genotypes of the 4G/5G polymorphism of PAI-I and DN.
	apoE				ε2/ε3/ε4	ε4	-	There were not association between genotypes of the ε2/ε3/ε4 polymorphism of apo-E and DN.
{Funk, 2005 #183}	*PAI-I*	DM2 Austrian	147	Cross-sectional Case-Control	4G/5G	4G	-	There were not association between genotypes of the 4G/5G polymorphism of PAI-I and DN.
Oxidative stress								
{Zanchi, 2000 #185}	*eNOS*	DM1 Caucasian	152/195	Cross-sectional Case-Control	T-786C	C	Recessive	Patients with CC genotype were at risk of DN [OR 2.8 (1.4-5.6))]
					I/D	D	Recessive	Patients with DD genotype were at risk of DN [OR 2.3 (1.3-4.0))]
					G894T	T	-	There was not association.
					$(CA)_n$	4a	-	There was not association.
				FBAT	T-786C+ID	CD	Haplotype	The D allele of the I/D polymorphism in intron 4 was preferentially transmitted to the offspring with advanced DN. Haplotype with allele C of T786C and D of the I/D polymorphism was preferentially transmitted to the offspring with advanced DN.
{Nagase, 2003 #130}	*eNOS*	Japanese ESRD different aetiologies	302/248 (231 Non diabetic ESRD, 71 diabetic ESRD)	Cross-sectional Case-Control	4a/4b	4a	Allelic	The allele 4a was associated with non diabetic ESRD.
			159/270 (17 hypertensive ESRD and 48 diabetic ESRD)		p.Glu298Asp	298Asp	Allelic	The allele 298Asp was associated with diabetic and non diabetic ESRD

Table 3.4. *Resume of candidate gene studies in DN (III).*

Author, year (ref)	GENE	POPULATION	SAMPLE SIZE (Cases/Controls)	STUDY DESIGN	GENE/POLYMORPHISM	RISK ALLELE	MODEL	Results
Oxidative stress								
{Rippin, 2003 #186}	*eNOS* *iNOS*	DM1 British	464/396	Cross-sectional Case-Control	4a/4b -/+	4a +	- -	There was not association with overt proteinuria. There was not association with overt proteinuria.
{Voronko, 2005 #187}	*PON1* *PON2*	DM1 Russian	62/68	Cross-sectional Case-Control	p.Gln192Arg p.Ala148Gly p.Cys311Ser	Arg Gly Ser	- - -	There were not association with DN. There were not association with DN. There were not association with DN.
{Lee, 2006#184}	*MnSOD*	DM2 Asian	127/244	Cross-sectional Case-Control	V16A	V	Alelic	The prevalence of the allele A was significantly lower in albuminurics than normoalbuminurics patients with type 2 diabetes.
Lipid metabolism								
{Werle, 1998 #193}	*apoE*	DM1 DM2 German	162 DM1 124 DM2	Cross-sectional Quantitative trait	ε2/ε3/ε4	ε2	Alelic	Carriers of the ε2 had significantly lower levels of CC than carriers of the other alleles. ε2 allele was independently associated with CC in the multiple linear regression model.
{Iakunina, 2005 #194}	*apoE* *apoB* *LPL*	DM1 Russian	62/68	Cross-sectional Case-Control	ε2/ε3/ε4 I/D p.Ser477Ter	ε3 I Ter	Allelic, recessive Alelic -	Carriers of the allele ε3 and genotype ε3/ ε3 had higher risk of DN than the other genotypes (OR 2.08 for the allele and 2.16 for the genotype) Carriers of the D allele had lower risk of DN than carriers of the I allele (OR 0.52) There was not association for this SNP of the LPL gene.
{Ng, 2006 #195}	*LPL* *apoE* *apoC3*	DM2 Asian	374/392	Cross-sectional Case-Control	p.S477X ε2/ε3/ε4 -455T>C p.S477X+ ε2/ε3/ε4 p.S477X+-455T>C ε2/ε3/ε4+-455T>C	S477 ε4 C	Doninant Doninant - Interaction	Genotypes with the allele 477X had lower risk of DN than the other genotype S477S (OR 0.66) Genotypes with the allele ε3 had lower risk of DN than the other genotype (OR 0.68) There was not association with DN The following combinations of genotypes were also of protection against DN (ε3 ε3 of apoE+ genotypes containing the allele 477X of the LPL, CC of apoC3+genotypes containing 477X allele of LPL and ε3 ε3 of apoE+ CC of apoC).
Growth factors								
{Patel, 2005 #200}	*TGFß1*	DM1 British	420/410	Cross-sectional Case-Control	p.Leu10Pro p.Arg25Pro p.Thr263Ile	Pro Pro Ile	Doninant - -	Genotypes of codon 10 were associated with type 1 DN. There was not association with DN. There was not association with DN.

Table 3.4. *Resume of candidate gene studies in DN (IV).*

Author, year (ref)	GENE	POPULATION	SAMPLE SIZE (Cases/Controls)	STUDY DESIGN	GENE/POLYMORPHISM	RISK ALLELE	MODEL	Results
Growth factors								
{McKnight, 2007 #202}	*TGFß1*	DM1 Irish	272/367	Cross-sectional Case-Control	-800G>A	A	-	There was not association with type 1 DN.
							-	There was not association with type 1 DN.
					-509C>T	T	-	There was not association with type 1 DN.
							-	There was not association with type 1 DN.
					+72InsC	C	-	There was not association with type 1 DN.
					+869T>C	C	-	There was not association with type 1 DN.
							-	There was not association with type 1 DN.
					+915G>C	C		
	TGFßR2				747C>G	G		
					1149G>A	A		
Glucose metabolism								
{Poirier, 2001 #213}	*RAGE*	DM1 Caucasian	199/193	Cross-sectional Case-Control	C-1152A	A	Allelic	The allele A of the C-1152 seemed to conferrer a weak protection against DN [OR 0.49 (0.25-0.94)].
					T-388A	A	-	There was not association with DN.
					G82S	S	-	There was not association with DN.
{Neamat-Allah, 2001 #205}	*ALDR*	DM1 DM2 Caucasians	471/494	Cross-sectional Case-Control 4 independent populations (2 DM1 and 2 DM2)	-106C>T	T	Allelic	The allele T was associated with DN in three of the four studied populations [OR 2.22 (1.69-2.94)]
				Meta-analysis for (CA)n	5'-(CA)n	z-2	-	There was not association with DN. The meta-analysis showed no association in type 2 diabetes and in type 1 DM is not clear.
{Pettersson-Fernholm, 2003 #212}	*RAGE*	DM1 Finish	675 (166 microalbuminurics, 325 with overt proteinuria and 184 with ESRD) /321	Cross-sectional Case-Control	-429T>C	C	-	No differences of genotypes between groups.
					-374T>A	A	Recessive	No differences of genotypes between groups. However in the subgroup of poor metabolic control (HbA1C>9.5), the genotype AA was less frequent in patients with proteinuria than in controls
{De Cosmo, 2009 #216}	*ENPP1*	DM2 Italian and White American	1392	Cross-sectional Three independent populations	p.K121Q	Q	Dominant	Genotypes containing the Q allele had higher risk of eGFR<60ml/min/1.73m^{2}than homozygotes p.K121K in two of the three studied populations [pooled OR 1.58 (1.2-2.1)]

DM1: type 1 diabetes; DM2: type 2 diabetes; ACE: Angiotensin converting enzyme; AGT: Angiotensinogen; AT1R: Angiotensin II receptor type 1; FABT: family based association test; PAI-I: plasminogen activated inhibitor; apoE: apolipoprotein E, MnSOD: manganese superoxide dismutase; eNOS: endothelial nitric oxide synthase; PON1: Paraoxonase 1; PON-2: Paraoxonase 2; LPL:lipoprotein lipase;apoC3:apolipoprotein C3; TGFß1: Tissular growth factor beta 1;TGFßR2: Transforming growth factor receptor 2; ADLR: Aldose reductase; RAGE: receptor for advanced glycation end products; ENPP1: Ectonucleotide pyrophosphatase/phosphodiesterase family member 1.

3.5.3. Linkage studies

Urine albumin: Krolewski et al[170] studying families with multiple relatives enriched for type 2 diabetes and most of them of Caucasian origin, found an heritability rate for the ACR running from 0.20 in the whole family to 0.39 in the group of relatives without diabetes[170]. Three quantitative trait loci were identified: One peak placed 36 cM in the long arm of chromosome 22, another peak to 69 cM in the q arm of chromosome 5 and the last peak to 169 cM in the q arm of chromosome 7[170]. Besides another additional peak was found in the p arm of chromosome 21 but only in the diabetic subgroup[170]. Maximum LOD score of 3.7 corresponded to the 22q loci[170] . Only in the 7q loci there was a strong candidate gene, the eNOS gene, that was previously associated with the risk of nephropathy by the same and another different research groups[170, 185, 220].

eGFR: In a Mexican-American population including diabetics and no diabetics patients and near 20% of hypertensives, Puppala et al[221] found a high heritability for the GFR estimated by Cockcroft-Gault or by Modification of Diet in Renal disease (MDRD) formula (0.40 and 0.36, respectively). They found suggestive association for eGFR by standard linkage analysis on chromosome 9q and another significant peak on chromosome 2q37.1 (LOD score 3.3) but only when using a genotype x diabetes interaction model[221]. These results reinforce the hypothesis of the genetic and environmental factors interaction.

The same group of Krowleski and colleagues[222] also studied the linkage with eGFR in 63 families with type 2 diabetes , the majority of them of European-American origin. They found a high heritability for the estimated GFR by different formulas (Cystatin C, MDRD, Cockcroft-Gault) especially in the group of diabetic subjects[222]. They found different linkage peaks than that observed for UAE. Maximum LOD score was found on chromosome 2q in diabetic relatives pairs (LOD score of 4.1) and 7p (LOD score 4.0) in all relatives pairs[222]. Additionally, another two linkage peaks, 10q, 18p, showed suggestive association in diabetic relatives pairs and other two, 3q and 11p, in nondiabetic relative pairs[222]. Within the 7p region lies the interleukine-6 gene and the neuropeptide Y (hNPY) which are known candidate genes for DN. The region 2q and 10q did not contain any candidate gene.

Taking into account these two studies, there might be an interaction between the diabetic environment and the presence of DN[221, 222]. It is not clear if the peak on chromosome 2q detected in both studies correspond to the same QTL for eGFR, although the two LOD score peaks are separated by 23 cM.

Diabetes nephropathy: As I mentioned before, it is important to remark the criteria to considerer DN in order to compare different studies as well the study population. The majority of the studies considerer as DN those subjects with both albuminuria-proteinuria and with ESRD.

In the study of Imperatori et al[84] using the albumin creatinine ratio (ACR) to define DN in type 2 diabetics Pima Indians, association peaks were found on chromosomes 7q, 3q, 9q and 20q. The

7q linkage peak contains a strong candidate gene, eNOS, and has been replicated latterly by another authors[170].

One of the most robust results were found in one linkage study for DN in type 2 diabetes performed in 18 Turkish families[223]. Cases included subjects with either proteinuria and ESRD[223]. A significant peak (LOD score 6.1) placed on chromosome 18q22.2-23[223] . Interestingly when they included patients with microalbuminuria as cases the magnitude of association increases from a LOD score of 6.1 to 6.6[223]. Moreover the authors were able to confirm the results in the previous described Pima Indian cohort[84, 224] although the association on chromosome 18 was not present in the initial analysis. Within this linkage peak lies the Carnosine Dipeptidase 1 gene (CNDP1) which has been associated with DN in several populations[225]. Moreover one variant of this gene seems to be of protection against DN[225, 226]. In contrast, one large case-control and follow-up study, trying to replicate the findings in Caucasians type 1 diabetics, did not find relationship between polymorphism of the CNDP1 gene and different stages of DN[227].

Another linkage study in an Afro-American population with type 2 diabetes only find modest association even after performed a multi-locus linkage analysis[151]. The highest LOD score belong to the 7p (LOD 1.43) for the single locus analysis and 16p for the multi-locus analysis (LOD 1.63). After performed an ordered subsets analysis (OSA) by age at diagnosis of ESRD, age at diagnosis of diabetes and duration of diabetes several peaks on chromosomes 3q, 7p and 18q increased the LOD score values and had a significant LOD score higher than 3[151]. Some of the associations are in concordance with other previous linkage studies[84, 223, 228]. Within the 3q peak is the ATR1 gene which is a strong candidate gene for DN, but it seemed not to be the responsible of the 3q linkage peak according to one study in Caucasian families with type 1 diabetes[228]. Adiponectin is another interesting candidate gene including in that linkage peak. One large study in African-American with type 2 diabetes including 851 with DN and 871 controls, found a significant association between the SNP *rs182052* in intron1 of the Adiponectin gene and type 2 diabetes and also with DN[229]. They did not detect association with the polymorphism in the promoter region described by Vionnet and colleagues[230].

Using 100 discordant sib-pairs (DSP)[231] from 83 families , Rogus et al[232] found a linkage peak not previously described on chromosome 19q (maximum likelihood score (MLS), 3.1) and another peak (MLS, 2.1) on chromosome 2q that overlapped one of the linkage peak for UAE found within the FIND study[233]. When considering separately proteinuria and ESRD, they found another four linkage peaks (1q (MLS=1.8), 20p (MLS=2.8), 3q (MLS=1.5 for proteinuria and MLS=1.1 for ESRD)[233]. These finding suggest that some of the susceptibility loci for UAE can be different than those for ESRD[233].

A resume of some of the main linkage studies for renal traits in type 1 and type 2 diabetes mellitus is shown in table 3.5 (I-VI).

Table 3.5. *Resume of linkage studies in DN (I).*

Study	Population	Sample size(N/Composition)	Study Design	Phenotype	Heritability	Chromosomal region
{Imperatore, 1998 #84}	Pima Indian (DM2)	59 families [135 diabetic siblings (98 with both siblings affected by nephropathy)].	Cross-sectional	DN defined as: UACR≥300 mg/g UPCR≥500 mg/g or ESRD	-	7q35 7q35 3q26.1-3q26.31 9q32 20p12.3-20p13
{Vardarli, 2002 #223}	Turkish (DM2) Pima Indian (DM2)	18 families (368 subjects): 61 normoalbuminurics, 28 proteinurics, 36 unknown status. 1338 (101 affected sibling pairs)	Cross-sectional Replication study	DN defined as: ACR≥300 mg/g PCR≥500 mg/g or ESRD (Patients with microalbumiuminuria were considered as unknown status.	-	18q22.3-23 18q22.3-23
{Bowden, 2004 #151}	African-American (DM2)	166 families (206 affected sibling pairs, 355 Affected individuals)	Cross-sectional	DN defined as: ESRD or CRF including: Cr≥2mg/dl proteinuria≥500mg/day uPCR≥0.5mg/g or uPCR≥100mg/dl on urine dipstick	-	7p 12 16p12.1 18q 14q32.13 21q22.3 3q13.32 7p15.3 18q 19p13.3
{Krolewski, 2006 #170}	Multiethnic, mainly European-American (DM2) (4 minority families)	63 extended families (5656 relative pairs: 1332 relative pairs concordant for diabetes, 1849 relative pairs concordant for the absence of diabetes and 2475 relative pairs discordant for diabetes)	Cross-sectional QTL	ACR (measured in a random urine sample and expressed as micrograms of albumin / milligrams of creatinine; ESRD were assigned ACR values of 2500 mg/mg)	0.23 (relatives with DM2) 0.39 (relatives without diabetes) 0.20 for the whole family	22q 5q 7q 21p (only in diabetics)

Table 3.5. *Resume of linkage studies in DN (II).*

Study	Chromosomal region	Main associated markers	LOD score	Results	Possible candidate genes
{Imperatore, 1998 #84}	7q35 7q35 3q26.1-3q26.31 9q32 20p12.3-20p13	D7S500 D7S1804 D3S3053 D9S302 GATA65E01	2.73 2.28 2.03 1.28 1.89	There were not markers with strong association with DN. A region in chromosome 7q showed suggestive association and contains interesting candidate genes. Another three genomic regions showed also suggestive association in 3q, 9q and 20p.	ADLR1(7q35) TRBC(7q35) NOS3(7q35) AGTR1 (3q)
{Vardarli, 2002 #223}	18q22.3-23 18q22.3-23	D18S469- D18S58 D18S469- D18S58	6.1 (6.6 when considering microalbuminurics as cases) 0.73	There was a significant linkage peak for DN in chromosome 18q22.3-23. The authors also were able to replicate the results in Pima Indian although that region did not showed association in the original study {Imperatore, 1998 #133}	ZNF236 (18q23) CNDP1 (18q22.3)
{Bowden, 2004 #151}	7p 12 16p12.1 18q 14q32.13 21q22.3 3q13.32 7p15.3 18q 19p13.3	D7S3051 GATA91H0 D16S769 D18S1364 D14S1434 D21S1446 D3S2460 D7S1802 D18S1364 D19S1034	1.43 (single-locus analysis) 1.06 (single-locus analysis) 1.00(single-locus analysis) 1.07(single-locus analysis) 1.63 (multi-locus analysis) 1.15 (multi-locus analysis) 4.55 (OSA by age at diagnosis of ESRD) 3.59 (OSA by duration of Diabetes to ESRD) 3.72 (OSA by age at diagnosis of diabetes) 3.13 72 (OSA by age at diagnosis of diabetes)	None of the selected markers got a LOD score higher than 2 . The maximum LOD score was in chromosome 7p. When using OSA, three locus showed strong association in 3q (early onset ESRD),7p (longer duration of diabetes) ,18q (early onset diabetes) an 19q (late onset diabetes).	ZNF236 (18q23) CNDP1 (18q22.3) AGTR1(3q24) Adiponectin (3q27)
{Krolewski, 2006 #170}	22q 5q 7q 21p (only in diabetics)	GATA11B12 GATA67D03 GATA30D09B GGAA3C07	3.7 3.4 (only when considering Caucasian families) 3.1 2.5	There were evidence for linkage with ACR in 22q, 5q and 7q. An additional locus 21p may exert its effect on urinary albumin excretion, but only in the presence of diabetes.	- - eNOS (7q36.1) -

Table 3.5. *Resume of linkage studies in DN (III).*

Study	Population	Sample size(N/Composition)	Study Design	Phenotype	Heritability	Chromosomal region
{Placha, 2006 #222}	Multiethnic, mainly European-American (DM2)	63 extended families (406 diabetics /428 without diabetes)	Cross-sectional QTL	eGFR- Cystatine C(CC)	All relatives: 0.28 ±0.06 Diabetics: 0.45 ±0.11 Non diabetics: 0.36 ±0.13	7p21 (all relatives) 2q (diabetics) 10q(diabetics) 18p11.22 (diabetics) 3q24(non diabetics) 11p11.2 (non diabetics)
				eGFR- Cockroft-Gault(CG)	All relatives: 0.31 ±0.06 Diabetics: 0.47 ±0.12 Non diabetics: 0.15 ±0.11	6p21.2 (all relatives) 2q (diabetics) 3q24(diabetics) 7q33 (non diabetics)
				eGFR - Modified Diet Renal Disease (MDRD)	All relatives: 0.28 ±0.07 Diabetics: 0.29 ±0.11 (eGFR-MDRD). Non diabetics: 0.22 ±0.12 (eGFR-MDRD).	6p21.2 (all relatives) 3q24 (diabetics) 19q (non diabetics)
{Iyengar, 2007 #233}	Multiethnic (DM1 and DM2) European-American (EA) African-American (AA) Mexican-American (MA) American-Indian (AI)	378 families (1227 individuals, 883 sibling pairs diabetes concordant)	Cross-sectional	DN based on: historical 24-h urine collection with ≥500 mg protein per 24 h or ≥300 mg albumin per 24 h, random protein-to-creatinine ratio ≥0.5 g/g, or random ACR ≥0.3 g/g. ESRD was defined as the need for chronic renal replacement therapy with either dialysis or renal transplantation	-	
		397 full sibling pairs for the qualitative trait (DN)	Qualitative trait DN			7q21.3 10p15.3 14q23.1 18q22.3
		883 sibling pairs for the quantitative trait (ACR)	Quantitative traits ACR			2q14.1 7q21.1 15q26.3

Table 3.5. *Resume of linkage studies in DN (IV).*

Study	Chromosomal region	Main associated markers	LOD score	Results	Possible candidate genes
{Placha, 2006 #222}	7p21 (all relatives)	D7S3047–D7S3051	4.0	In the whole population it was found a strong Linkage peak in 7p.	Il-6 (7p15.3) NPY (7p15.3)
				There was another strong linkage peak in 2q	-
	2q (diabetics)	D11S1993	4.1	but only in diabetics subjects and	RF1 (10q)
	10q(diabetics)	D10S2470–D10S677	3.6	a suggestive peak in 10q also in diabetics. These	
	18p11.22 (diabetics)	D18S843	2.2	facts reinforce the hypothesis of an interaction between genetic and DM2.	
	3q24(non diabetics)	D3S1744	2.2	In non diabetics subjects there were only suggestive	
	11p11.2 (non diabetics)	D11S1993	2.1	peaks in 3q and 11p.	
	6p21.2 (all relatives)	D6S1548	1.7		
	2q (diabetics)	D11S1993	2.0		
	3q24(diabetics)	D3S1744	2.6		
	7q33 (non diabetics)	D7S500	1.3		
	6p21.2 (all relatives)	D6S1548	1.8		
	3q24 (diabetics)	D3S1744	2		
	19q (non diabetics)	D19S890	1.2		
{Iyengar, 2007 #233}			LOD/p-value	There were significant linkage peaks for DN in 7q, 10p, 14q and 18q and in chromosome 2q, 7q and 15q for DN.	
					PON1 y PON2 (7q21-22)
	7q21.3	D7S2212-D7S821	3.9/6e-005 (AA)	It is possible that the susceptibility loci for DN were	NRP1(10p11.22)
	10p15.3	D10S1435-D10S189	1.5 /1.4e-004(AI)	different from the loci controlling the UAE.	RF5(10p)
	14q23.1	D14S587-D14S588	3.3/ 7.23e-005(AI)		CNDP1 (18q12.3)
	18q22.3	D18S1371-D18S1390	2.0/1.61e-003 (AII)		
	2q14.1	D2S410-D2S1328	3.7/8.24e005 (AI)		IL-1B (2q14)
	7q21.1	D7S3046-D7S2212	3.7/3.76e-005 (EA)		PON1 y PON2 (7q21-22)
	15q26.3	D15S657-D15S642	2.8 /1.85e-004(AA)		IGF1R (15q26.3)

Table 3.5. *Resume of linkage studies in DN (V).*

Study	Population	Sample size(N/Composition)	Study Design	Phenotype	Heritability	Chromosomal region
{Puppala, 2007 #221}	Mexican-American (DM2)	39 large families (741 individuals: 130 diabetics and 323 no diabetics; 60 with HTN in the diabetic group and 22 in the non diabetic group)	Cross-sectional	eGFR	0.40±0.09 for eGFR by Cockkroft-Gault (GC) Formula in the whole sample. 0.36±0.09 for eGFR by MDRD formula in the whole sample 0.54 for eGFR (CG) in the diabetic group 0.43 for eGFR (4VMDRD) in the diabetic group 0.56 for eGFR (CG) in the non diabetic group 0.36 for eGFR (MDRD) in the non diabetic group	9q21 2q36.3 3p26.3 3q23-24 4p15.1-p13 8q24.23 11q24.23-q25 14q32.2-q32.33
{Rogus, 2008 #232}	Caucasian (DM1)	83 families: 80 parents, 96 siblings with type 1 diabetes but without diabetic nephropathy, 43 diabetic siblings with persistent proteinuria, and 44 diabetic siblings with ESRD; 130 sibpairs concordant for type 1 diabetes and 100 sibpairs discordant for DN	Cross-sectional	DN based on: Persistent proteinuria [>250 mg/g (men) or >355 mg/g (women) or by a multistix reagent strip >2] ESRD or renal transplant	-	19q13.42 5q22.1 10q26.11 2q13 17p13.1 20p12.1 1q44

Table 3.5. *Resume of linkage studies in HTN (VI).*

Study	Chromosomal region	Main associated markers	LOD score	Results	Possible candidate genes
{Puppala, 2007 #221}	9q21	D9S922 -D9S1120	2.9 (model 1)*	There were a high heritability for both eGFR by CG	-
			2.4 (model 2)** eGFR-CG	or MDRD in the whole sample, diabetics and non	
		D9S922–D9S1120	2.6 (model 1) eGFR-MDRD	diabetics.	
				A strong linkage peak (model 1) was found in	
	2q36.3	D2S1363	2.7 (model 1)	chromosome 9q for eGFR by CG and MDRD.	CAPN10 (2q35-q37.3)
			3.3 (model 2) eGFR-CG	There were not known genes for renal function under	IRS1 (2q35-q37.3)
				that linkage peak in 9q.	COL4A3 (2q35-q37.3)
	3p26.3	D3S2387	1.4 (model 1)	The linkage peak in chromosome 2q36.3 was wide	COL4A4 (2q35-q37.3)
			1.7 (model 2) eGFR-CG	considering genetic x diabetes mellitus interaction.	PTPRN (2q35-q37.3)
				Under these peak there are several candidate genes.	SMARCAL1 (2q35-q37.3)
	3q23-24	D3S1764–D3S1744	1.4 (model 1) and		
			1.6 (model 2) for eGFR-CG		AGTR1 (3q24-25)
	4p15.1-p13	D4S2632–D4S1627	1.3 (model 1)		
			1.2(model 2) eGFR-CG		
	8q24.23	D8S373	1.2 (model 2)eGFR-CG		
	11q24.23-q25	D11S912–D11S968	2.4 (model 1)		
			1.9 (model 2) eGFR-CG		
	14q32.2-q32.33	D14S1426–D14S1007	1.7 (model 1) and		
			1.2 (model 2) for eGFR-CG		
{Rogus, 2008 #232}			MLS values:	There was a strong linkage peak for DN in 19q and	
	19q13.42	rs306450	3.1	another suggestive peak on chromosome 2q.	
	5q22.1	rs27342	2.7	The 1q peak was not present in DSP with	
	10q26.11	rs1467813	2.5	proteinuria	Several genes of the interleukin 1 family (2q13)
	2q13	rs895415	2.1	The 20p peak was not present in ESRD DSP	
	17p13.1	rs1047365	1.9		
	20p12.1	rs775133	1.8		
	1q44	rs987179	1.6		

DM1: type 1 diabetes; DM2: type 2 diabetes; UACR: urinary albumin creatinine ratio; UPCR: urinary protein creatinine ratio; ESRD: end stage renal disease; ADLR1: aldose reductase; TCRBC: T cell receptor β chain; NOS3: endothelial nitric oxide synthase 3 ; AGTR1. angiotensin II receptor 1; OSA; Ordered subsets analysis; ZNF236: Kruppel-like zinc-finger gene 236; CNDP1: Carnosine Dipeptidase 1 gene; QTL: quantitative trait loci; eNOS: endothelial nitric oxide synthase; CC: cystatin C; CG: Cockroft-Gault; MDRD: modified diet renal disease; eGFR: estimated glomerular filtrate rate; Il-6: interleukine 6; NPY: neuropeptide Y; RF1: rat renal failure 1 gene; PON1: paraoxonase 1; PON2: paraoxonase 2; NRP1: neuropilin 1; RF5: rat renal failure 5 gene; IGF1R: Insulin-like growth factor 1 receptor Precursor; CAPN10: calpain 10; IRS1: insulin receptor substrate; COL4A3: collagen type IV alpha-3; COL4A4: collagen type IV alpha-4; PTPRN: a member of the protein tyrosine phosphatase (PTP) family; SMARCAL1 :SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A-like protein 1 IL1B: interleukin 1 beta; ; MLS: maximum likelihood score; DBP: discordant sib-pairs
* Model 1: without considering genetic x DM interaction; ** Model 2. taking into account the GxDM2 interaction.

3.5.4. Whole genome association studies and combined strategies

The genetics of kidney in Diabetes (GoKind) study is a large study using different strategies in order to unravel the genetic of nephropathy in type 1 diabetes[234]. Within this project are included several study design based on case-control and family trios[234]. Cases include patients with macroalbuminuria, proteinuria or ESRD and the controls are all normoalbuminurics after more than 10 years of follow up[234]. One first genome wide scan with a low density panel of markers did not reveal associated SNPs after correcting for multiple testing. Two of the most associated SNPs were located in the pleckstrin homology domain containing, family H (PLEKHH2) gene which is expressed in the podocytes of the glomeruli and therefore is an interesting candidate gene. Using Tag-SNPs and the same GoKind cohort[235], several SNPs in the PLEKHH2 were analyzed[235]. Between all the selected SNPs, the allele *T* of one SNP in the promoter region (*rs11886047*) showed association with DN by transmission disequilibrium test (TDT)[235]. The fact that there were a higher frequency of heterozygotes CT in cases than in controls seems to reinforce the results[235]. 1177 type 1 diabetes from this GoKind population and 850 African-American with type 2 diabetes were used to assess the association of a new candidate gene, the transient receptor potential cation channel subfamily C, member 1 (TRPC1), identify in one of the linkage peak (3q22-24) in both type 1 and type 2 diabetes[236]. There were not association between SNPs of the TRPC1 gene and DN, but the expression of this gene seems to be reduced in animals with DN[236]. The authors suggest that the alterations in the TRPC1 expression can be a late phenomenon in DN[236].

Ewens et al[237] evaluated the association of variants in 115 candidate genes with DN by TDT in 72 families with type 1 diabetes of European ancestry. They found association with genes involve in the kidney function such as AGTR1, Aquaporin1 (AQP1), LPL, genes coding extra-cellular matrix components, transcription factors and growth factors[237]. Probably many of the association are false positive because of the lack of power of the study. The most associated SNPs were within the B-cell leukaemia/lymphoma 2 (bcl-2) gene (p-value=0.001) and within the Collagen type 4 Alpha 1 (COL4A1) gene (p-value=0.0002)[237]. The former gene lies close to a region identify by linkage studies[223] and the second one was previously associated to DN with contradictories results[238, 239].

Regarding the type 2 diabetes, the Family Investigation of Nephropathy in Diabetes (FIND)[240] study is another multicenter study which is using different strategies to discover susceptibility variants for DN in type 1 and type 2 diabetes. Four ethnics groups are included: European-American, African-American, Mexican-American, and American-Indian. Three are the main used strategies: genome wide linkage study[241], mapping by admixture linkage disequilibrium (MALD)[242] and test to detect the association between alleles and disease within the population. Cases also includes subjects with macroalbuminuria, proteinuria or ESRD[240]. One initial linkage study within these populations revealed four linkage peaks for DN: 7q21.3, 10p15.3, 14q23.1, 18q22.3 after pooling all the p-values in the four populations by Fisher´s method[233]. The linkage peaks on chromosome 7q is

mainly due to the African-American subpopulation, the 10p and 14q are mainly due to the American-Indian and the 18q peak mainly due to the European-American subpopulation[233]. The quantitative trait linkage analysis for ACR showed a linkage peak in 2q14.1 in American-Indian, 7q21.1 in European-American and 15q26.3 in African-American families[233]. The linkage study for eGFR as a quantitative trait by the MDRD equation, revealed several suggestive peaks, using multipoint IBD, on chromosomes 1q43, 7q36.1, 8q13.3, 11p15.1, 18q13-21, and 20p12.2, after adjusted by diabetes duration and ACE inhibitor/ARB use. In the Mexican-American cohort which compromises the majority of the families, the 8q and 18q peaks reached the Langer-Kruglyak[141] significance for linkage [243].

A GWAS with around 100000 SNPs was performed in Japanese population with type 2 diabetes[244]. The study included 94 type 2 diabetic cases with DN and retinopathy and 94 controls with retinopathy but without nephropathy. A great number of loci were identified and were selected for further evaluation in a large number of subjects[245]. There was a strong association between DN and SNPs in the following genes: Solute carrier family 12, member 3 (SLC12A3), Engulfment and cell motility 1 (ELMO1) and neurocalcin D (NCALD). Although the authors performed several in vitro studies[246-248] to asses the role of these gene in the pathogenesis of DN, the mechanisms for renal damage associated with variants in these genes is unknown.

As I said before, the most associated SNPs (p-value <0.01) were selected to be replicated in a larger sample that the previous one including 466 cases and 266 controls[246]. One SNP within an intronic region of the ELMO1 gene was associated with DN in that Japanese population[246]. To asses the role of that gene in the development of DN, they study the gene expression in normal and diabetic mice[246]. That expression was mainly observed in glomerular and tubular epithelial cells and was increased in the kidney of diabetic mice[246]. They also demonstrated high mRNA gene expression in cell cultures under high glucose conditions and an increase on the expression of extracellular matrix protein genes such as TGF-β1 in cells overexpressing ELMO1[246].

Hanson et al[249] in a pooling based genome wide scan[250, 251] for ESRD in type 2 diabetes Pima-Indian found association with markers in 8q24.21[249]. This region contains the plasmocytoma variant translocation gene (PVT1) a new candidate gene for DN. This gene is highly expressed in the kidney[252] and could easy the proliferation of the mesangial cells which usually occurs in the DN.

A resume of the great scale candidate gene study of Ewens et al[237] and some of the main genome wide association studies for renal traits in type 1 and type 2 diabetes is shown in table 3.6 (I,II,III).

Table 3.6 *Resume of a great scale candidate gene study for DN (I).*

Study	Population	Sample size (N)	Study Design/Platform	Phenotype	Chr region	Main associated markers	p-values	Results	Possible candidate genes
{Ewens, 2005 #237}	European-American (DM1)	72 families(68 parent-child trios and 4 multiplex families).	Comprehensive evaluation of 115 candidate genes for DN (1 SNP every 20kb) by TDT/ mainly Applied Biosystems Taqman SNP Genotyping Assays	DN based on: UACR≥300mcg/min or ESRD	18q21.33	rs2062011(BCL2)	0.001	In all the genes, the only SNP which was significant beyond the nominal p=0.001 was rs679062 in COL4A1. nominally significant p-values were obtained for polymorphism 20 genes but some of them could be in fact false positive because of the multiple comparison problem. Three of the associated SNPs showed transmission distortion in CEPH control families: rs560807 in CAT, rs11697325 in MMP9 and rs6792117 in TGFBR2.	BCL2(18q21.33)
					18q21.33	rs12457700(BCL2)	0.006		
					18q21.33	rs14810318BCL2)	0.009		CAT(11p13)
					11p13	rs1049982(CAT)	0.006		
					11p13	rs560807(CAT)	0.044		LAMA4 (6q21)
					6q21	rs3734287(LAMA4)	0.016		
					3p24.1	rs6792117 (TGFBR2)	0.024		TGFBR2 (3p24.1)
					1p22.1	rs12756024 (TGFBR3)	0.018		TGFBR3 (1p22.1)
					3p21.31	rs1800668 (GPX1)	0.022		GPX1 (3p21.31)
					1q25.3	rs20557(LAMC1)	0.026		LAMC1 (1q25.3)
					15q22.23	rs12594610 (SMAD3)	0.033		SMAD3(15q22.23)
					15q22.23	rs4776890 (SMAD3)	0.046		USF1 (1q23.3)
					1q23.3	rs2516839 (USF1)	0.047		AQP1 (7p14.3)
					7p14.3	D7S526 (AQP1)	0.027		IGF1 (12q23.2)
					12q23.2	MFD1 (IGF1)	0.047		TIMP3 (22q12.3)
					22q12.3	D22S280 (TIMP3)	0.048		COL4A1 (13q34)
					13q34	rs614282 (COL4A1)	0.002		
					13q34	rs679062 (COL4A1)	4e-4		AGTR1(3q25.1)
					3q25.1	D3S1308 (AGTR1)	0.001		LPL (8p21.3)
					8p21.3	rs320 (LPL)	0.005		
					8p21.3	rs326 (LPL)	0.011		
					8p21.3	rs13702 (LPL)	0.004		PRKCB1 (16p12.1
					16p12.1	rs1015408 PRKCB1)	0.025		NRP1 (10p11.22)
					10p11.22	rs869636 (NRP1)	0.047		
					10p11.22	rs2804495 (NRP1)	0.027		
					17q12	rs2688 (HNF1B1)	0.029		HNF1B1 (17q12)
					16q24.3	rs4673 (CYBA)	0.032		CYBA (16q24.3)
					20q13.2	rs11697325 (MMP9)	0.029		MMP9 (20q13.2)

Table 3.6 *Resume of genome wide association studies in DN (II).*

Study	Population	Sample size (N)	Study Design/Platform	Phenotype	Chr region	Main associated markers	p-values	Results	Possible candidate genes
{Maeda, 2004 #244} {Maeda, 2007 #245}	Japanese (DM2)	94 cases/94 controls	Case-Control/ 100000 SNPs Were randomly selected from the IMS-JST Japanese SNP database and were genotyped by invader assay combined with multiplex PCR.	DN based on: DM2 with retinopathy Plus UAE≥ 200 mcg/min or ACR ≥300 mg/g or ESRD under replacement treatment	16q13 7p14.1-14.2 8q22.3	rs2289116 (SLC12A3) rs741301 (ELMO1) rs131863 (NCALD)	2e-005 8e-006 4e-005	Nine SNPs showed a strong association with P <0.0001; 1625 had a p-value<0.01. All these SNP were selected for replication.	SLC12A3 (16q13) ELMO1 (7p14.1-2) NCALD (8q22.3)
{Tanaka, 2003 #248}	Japanese (DM2)	553 cases/317 controls (replication study SLC12A3)	Replication study for SLC12A3 (31 SNP were investigated Along the gene: one SNP from the 5'flanking region, one in the first intron, 21 in other introns, and three in exons. Additionally three exonic SNPs as well as two in the first intron were assessed by direct sequencing).	DN	16q13	rs11643718 (SLC12A3)	2e-005	They found association with 4 of the 31 SNPs in that gen analyzed, especially with the functional mutation Arg913Gl in exon 23.	SLC12A3 (16q13)
{Shimazaki, 2005 #246}	Japanese (DM2)	640 cases/426 controls (replication study ELMO1)	Replication study for ELMO1(516 SNP were investigated along the gene: 448 SNPs, 49 insertion/deletion polymorphisms, 18 tandem repeat polymorphisms, and 1 other polymorphism)	DN	7p14.1-14.2	rs741301 (ELMO1)	8e-006	They found one strong association with one SNP in intron 18 (+9170A>G). They also found association with two haplotypes but this association was much more weaker than that for the individual SNP.	ELMO1 (7p14.1-2)
{Hanson, 2007 #249}	American-Indian (DM2)	105 cases/102 controls (included 53% with ACR 30–299 mg/g)	Case-Control in a pooling based GWS/ Affymetrix 100K Human Mapping set	ESRD	8q24.21 8q24.21 8q24.21 8q24.21	Sliding window analysis rs2720709 rs1499368 rs4492334	0.002 2.1e-005 1.3e-003 0.09	The most associated Genomic area was located in chromosome 8q24.21 overlapping the PVT1 gene. Several SNPs within this Gene were associated with ESRD.	PVT1 (8q24.21)
		105 cases/102 controls		Further evaluation of the PVT1 gene with 101 SNPs along the gene.	8q24.21 8q24.21 8q24.21	rs2648875 rs1499373 rs2648875	2e-006 3e-006 3.6e-006	A comprehensive analysis of this gene demonstrated that the main marker for this association was rs2648875 due to the individual SNP analysis and the haplotype analysis.	

Table 3.6 *Resume of genome wide association studies in DN (III) .*

Study	Population	Sample size (N)	Study Design/Platform	Phenotype	Chr region	Main associated markers	p-values	Results	Possible candidate genes
{Greene, 2008 #235}	Mainly European-American (DM1)	112 cases/148 controls	Case-Control (HLA DR3/4 subset) / Affymetrix 10 K Xba V 2.0 mapping array	DN based on: UACR≥300mcg/mg or ESRD	2p21	Single: rs1368086	2.12e-05 (allelic test) 8.87e-05 (genotypic test)	No single SNP reached the genome wide significance after correcting for multiple testing. The authors did not find association with the previous SNPs within the familial study but they found another SNP within the PLEKHH2 and several haplotypes including the previous markers associated with DN. In a larger sample they tried to replicate the association with the SNP significantly associated in the case trio study. They found significant differences between genotypes but only when comparing the heterozygotes with the other homozygotes (Underdominance model or heterozygote disadvantage). This last association was stronger when they compared the case with the long duration controls.	PLEKHH2 (2p21)
		246 case trio families	Trio families/TDT (the two SNPs associated in the GWAS and another Tag SNPs within the PLEKHH2 gene were studied in the case trios by TDT)		2p21	rs725238	8.96e-04 (allelic test)		
					2p21	rs11886047	3.68e-03 (genotypic test)		
						Haplotype:			
					2p21	TC (rs7558302-rs11886047)	0.0307		
					2p21	AGTA(rs919694-rs1368086-rs4953011 - rs725238)	0.0326		
		601 cases/577 controls (replication)	Case/Control (replication for the SNP associated by TDT (rs11886047)		2p21	rs11886047	0.00343 (genotypic test) 0.00793 (Underdominance model TT + CC vs. CT) No differences for the allele frequencies		

DM1: type 1 diabetes mellitus; DM2: type 2 diabetes mellitus; DN: diabetes nephropathy ;UACR: urinary albumin creatinine ratio; ESRD: end stage renal disease; PLEKHH2: pleckstrin homology domain containing, family H; BCL2: B-cell leukemia/lymphoma 2; CAT: catalase; LAMA4: laminin alpha 4; GPX1 glutathione peroxidase 1; LAMC1: laminin gamma 1; SMAD3: mothers against DPP homolog 3; USF1: Upstream transcription factor 1; AQP1: aquaporin 1; TIMP3: tissue inhibitor of metalloproteinase 3; IGF1: Insulin-like growth factor IA Precursor; COL4A1: Collagen, type IV alpha 1; AGTR1: angiotensin II receptor type 1; LPL: lipoprotein lipase; PRKCB1: protein kinase C beta 1; NRP1: Neuropilin 1; HNF1B: Hepatocyte nuclear factor 1-beta; CYBA: Cytochrome b-245 light chain; MMP9: matrix metalloproteinase 9; SLC12A3: Solute carrier family 12 member 3; ELMO1:

3.5.5. Summary of genetics of microalbuminuria in diabetes mellitus

Familial aggregation, epidemiological and animal studies support the genetic contribution for kidney damage in type 1 and type 2 diabetes[76, 77, 83, 85, 86].

As I said in the case of hypertensive nephropathy, great efforts have been made in this field but we still are far away to know which major genetic variants are causing the susceptibility to develop nephropathy in diabetes.

In general, candidate gene strategy has given contradictory information. Although there are some variants which have been replicated, the majority of them were not or only showed association in certain subgroup of patients selected by intrinsic or extrinsic factors. Between the different physiological pathways that have been explored, the RAAS system as in the case of HTN, is probably involved. We can not forget that this system has been largely studied and that there is a bias to publish only positive results. These bias for positive results and the difficulties to compare different studies, make doubtful the results of the two meta-analysis for the *I/D* polymorphism in ECA gene[174, 175]. The *D* allele of this polymorphism has been related with the blood levels of ECA[157] what increase the possibility of a functional role for this variant. Although the meta-analysis are not concluding, one large case control study in three different European type 1 diabetic populations has given credit to the role of the *D* allele, alone or in haplotypes with others alleles, in the development of albuminuria [158]. The evidence for other variants of the RAAS genes is less clear although there are several papers which support the interaction between different variants of this system[176-179].

Linkage studies have given credit to some of the previously described candidate genes and have also identified some others genes. The high heritability for ACR in this kind of studies suggest an interaction between genetic and environmental factors[77, 168-170].

Some of the linkage peaks deserve special attention because their replication in different studies and their association with different renal traits. This is the case of the chromosomal region 3q. This region has been associated with DN especially in Caucasian type 1 diabetes[228, 237, 253]. In type 2 diabetes there has been association with DN defined by ACR in Pima Indian[84] and with eGFR in African-American [151]. Initially it was though that the AGTR1 gene was the responsible for this peak[84] , but ulterior studies have rule out this hypothesis[228]. It seems that the adiponectin gene may be the responsible for this peak according to the study of Vionnet N et al[230]. Adiponectin is though to be involved in the atherosclerosis process and therefore in the glomerular damage. This adipocytokine can blocked several mechanism involve in the atherogenesis[254]. It is supposed to reduce the adhesion of molecules to endothelial cells, the oxidative stress by stimulation of eNOS and the vascular smooth cells proliferation[255]. Also in 3q is the TRCP1, but this gene seems not to be involved at least in the initial phases of DN[236].

Chromosome 7 also has been repeatedly associated with DN especially in type 2 diabetes. The region 7q was associated with DN in type 2 diabetic Pima-Indian[84] and with DN and GFR, especially in Mexican-American, within the FIND study[233, 243]. The fact that this chromosomal region contains interesting candidate genes reinforces its possible role in the pathogenesis of DN at least in type 2 diabetes. Within these genes are the ALDR1 and eNOS which have shown association with nephropathy in candidate genes studies[130, 185, 186, 205]. The short arm of chromosome 7 have also been linked with eGFR in Caucasian type 2 diabetics[222] and with ESRD in African-American type 2 diabetes[151]. Within the genes which can be responsible for these peaks are the Il-6 and neuropeptide Y (hNPY)[256, 257]. Also in 7p lies the ELMO1 gene which were linked with DN in Japanese with type 2 diabetes according to the GWAS performed by Shimazaki A and colleagues[246]. Their posterior functional analysis seemed to confirm that results[246]. Probably, the most strong association for DN correspond to the 18q locus if we take into account the Lander and Kruglyak scale[141]. This locus was firstly described in Turkish families with type 2 diabetes[223] and replicated in Pima-Indian also with DM2[84, 224]. A similar peak was found for the association with both DN in different populations mainly European-American and with eGFR in Mexican-American within the Find study[233, 240]. CNDP1 gene which codifies the enzyme carnosinase could be the responsible for this linkage peak[225, 226]. This enzyme degrades the dipeptide carnosine which can act as an ACR inhibitor[258, 259], natural reactive oxygen species scavenger[260] and advanced glycation end products (AGEs) breaker, therefore can protect the kidney in diabetic subjects. Although it is a plausible candidate gene at least in type 2 diabetes, a recently published paper in type 1 diabetes failed to find association between this gene and nephropathy[227].

The GWAS have also been useful to discover new potential genes associated with DN. Between these genes are the PLEKHH2[234, 235]. The protein coding by this gene has been demonstrated to be expressed in the glomerular podocytes[261] and therefore it constitutes an interesting research pathway to follow.

Another three interesting genes such as ELMO1, SLC12A3, NCALD, and PVT1 have been identify in Japanese population and Pima-Indian with type 2 diabetes[244-246, 249]. These genes could be involved in the mesangial proliferation and fibrosis associated with DN[247] Further in vitro and replication studies could clarify in the future if they are or not susceptibility genes for DN.

In this last part I have tried to make a brief summary of some of the most important papers published in this field in diabetes. In spite of the research which has been made what are the variants and genes mainly implicate in the susceptibility for DN is not clearly defined.

As I said before, probably well design multicenter studies using different strategies in very large populations can help to clarify the current information in this topic.

Hypothesis and objectives

4. HYPOTHESIS AND OBJECTIVES

4.1. Hypothesis

Some evidences exist about the heritability of renal dysfunction independent in part of the traditional risk factors involved in the production of renal damage, high blood pressure and abnormalities of the glucose metabolism among others. The most used surrogate marker for renal dysfunction is the increment in urinary albumin excretion. Mainly candidate gene and linkage analysis has been used to identify the potential genetic factors associated with the urinary albumin excretion with variable success.

Our hypothesis is that if there is a genetic basis for increment of urinary albumin excretion in essential hypertension subjects, the genome wide association strategy, by means of high-density microarrays with hundreds of thousands SNPs, can help us to know what regions are involved and what candidate genes fall in those regions.

4.2. Objectives

4.2.1. Primary

- Identify significantly different genotype or allele frequencies between hypertensives with or without persistent microalbuminuria by means of a dense panel of SNPs all across the genome.
- Identify in the whole genome possible quantitative trait loci for UAE.

4.2.2. Secondary

- Identify haplotypes of risk of microalbuminuria in the associated regions.
- Identify which haplotypes can act as a QTL for UAE.
- Identify candidate genes for albuminuria and microalbuminuria in HTN within the associated chromosomal regions.
- Assemble our results with those previously published in the literature to construct a SNPs panel for assessment the risk to develop an increment in urinary albumin excretion.

Materials and methods

5. METHODOLOGY

5.1. Study design

The study design is a nested case-control study. Both cases and controls belong to a hypertensive cohort which was previously used to identify what clinical factors influence the development of microalbuminuria in HTN in a prospective way[18]. The patient recruitment was made between the years 1988 and 2000 in the hypertension unit of two hospitals: Clinical Hospital of Valencia and the Sagunto's hospital.

5.1.1. Inclusion criteria

The inclusion criteria at the beginning of the follow-up were:

1. Caucasian race
2. Age within 25 and 50 years old
3. Blood pressure levels within the normal-high or grade 1 hypertension according to the European societies of hypertension and cardiology. This is a systolic blood pressure (SBP) within the range 130-159 mmHg and diastolic blood pressure (DBP) within the range 85-99 mmHg in at least three visits which were spaced by one month among them and after ruling out secondary hypertension.
4. Normal urinary albumin excretion (UAE) (< 15 mg/day)
5. Never treated with hypertensive drugs
6. Informed consent

5.1.2. Exclusion criteria

Main exclusion criteria were:

1. Secondary hypertension
2. Nephropathy or diabetes mellitus
3. Urinary tract infection when UAE was determined
4. Lack of reproducibility among different UAE measurement
5. Blood pressure levels outside the pre-specified range of values

After the recruitment the patients were controlled yearly in the outpatient department. UAE was assessed in each one of the visit during the follow-up. Patients were withdrawn from the study if they developed microalbuminuria (UAE within the range 30-300 mg/day).

All the patients were treated with lifestyle modifications and antihypertensive drug treatment when needed. Therapeutic actions included: physical exercise, low sodium and saturated fats intake and antihypertensive drugs in case the previous actions failed. Within the antihypertensive drugs we used whichever of the following: ß o α –blockers, angiotensin converting enzyme (ACE) inhibitors, angiotensin II receptor antagonist (ARA II) and calcium channel blockers. Diuretics such as hydrochlorothiazide or other drugs were added if BP levels remained uncontrolled. Yearly the following actions were performed: blood pressure levels, 24 hour UAE, and biochemical profile.

5.2. Phenotyping

For the present study cases and controls have been selected from the above described cohort according to the UAE during the follow-up:

5.2.1. Controls

Subjects with a mean for the 24 hour UAE less than 30 mg/day (normoalbuminurics).

5.2.2. Cases

Subjects with a mean for the 24 hour UAE ≥ 30 mg/day and < 300 mg/day (microalbuminurics) or >300 mg/day (albuminurics).

As I mentioned above, the study subjects were divided into two groups: normoalbuminurics, if the UAE is less than 30 mg/day and microalbuminurics, if the UAE is ≥30 mg/day and < 300 mg/day. We also included 16 patients with UAE ≥ 300 mg/day in order to increase our sample size and our statistical power. The patients were extracted of a hypertensive cohort with similar characteristics at the time of recruitment: age, blood pressure levels and naïve regarding to the treatment. In this way we can see in a prospective way which are the factors influencing the development of microalbuminuria in HTN.

Among all the patients, some of them developed microalbuminuria (cases) whereas others did not develop it (controls). This design enables to reduce the misclassification bias. Besides, the fact that we have used the mean of two 24 hour urinary samples for each subject reduces the possibility of false positive or negative regarding to the 24 hour variability and circadian rhythm of the UAE. We even considerer a third sample when the differences in UAE between the two samples were greater than 25% or if the sample was not well picked up. The right classification of the subjects in cases and controls is a key factor in an association genetic study. Another thing to take into account is that the majority of our controls (>80%) remained stable regarding to the UAE with very low levels (< 15 mg/day) so they can be considerer as hyper-controls. The use of hyper-controls is a way to reduce the misclassification because they probably will not developed microalbuminuria in the future.

The basis principle of our study is that the genetic susceptibility threshold is different in cases than it is in controls. The well-known factors: age, BP levels, glucose profile and time of evolution of hypertension, would result in renal damage depending on the individual genetic susceptibility.

After the selection according to the case-control status (2 controls for each case), peripheral blood was extracted in order to get the deoxyribonucleic acid (DNA) for the genotyping. The genotyping included more than 250000 markers spaced along the whole genome.

5.3. Sample size

In this kind of studies, this is, genetic association studies with high-throughput platforms and thousand or millions of SNPs, it is quite difficult to determine the minimum sample size to detect true associations. In general the bigger it is the sample size the greater the possibility to find true significant associations. Small sample sizes can generate multiple false signals across the entire genome. That is not always the case. For example, one study with the Gene-Chip Human Mapping 500K with 50 samples, found a very strong association with one genomic area which disappeared when the sample size was increased.

The sample size that it needs to detect association between a genetic variant and a trait or disease, depends on: variant frequency, the risk associated with that variant and the linkage disequilibrium (LD) between that variant and a close marker. If we considerer that the LD magnitude between the marker an the variant is d^2, then the sample size to detect association with that marker is approximately N/ d^2 [262] . These theoretical principles explain why it is so difficult to calculate the minimum sample size, because we do not know a priori the factors to determine it. So we have to speculate and calculate it under different scenarios. Huge sample sizes, which are difficult to obtain in clinical studies, can be avoided if the density of the SNPs along the genome is as high as 500000 SNPs with a mean space between them of 6 kb across the genome. This is based on the estimation made by Kruglyak and cols[262]. The mean space between SNPs in the genotyping platform “GeneChip® Human Mapping 500K Array Set” is 5,6 kb.

5.4. Clinical and laboratory procedures

5.4.1. Blood pressure measurements (BP)

It was assessed in a quite place after 5 minutes resting and according to the guidelines of the British Society of hypertension (http://www.bhsoc.org/bp_monitors/BLOOD_PRESSURE_1784b). Systolic and diastolic blood pressure are the phases I and V of Korotkoff, respectively.

5.4.2. General analytics

Blood samples were extracted early in the morning after 8 hours fasting. Serum biochemistry was assessed by mean of a multichannel auto-analyzer SMAC (Technicon Instruments Cor, Tarrytown, New York USA). Plasma glucose was assessed by the glucose oxidase method (Beckman Glucose Analyzer, Beckman Instruments, Fullerton, Ca, USA). Total cholesterol and triglycerides were assessed by an enzymatic method (Roche Diagnostics). Blood for the genotyping was taken into tubes 15% of ethylenediaminetetraacetic acid (EDTA) and was kept at 4ºC to process in 5 days time Those samples which were not processed in five days after the extraction were frozen at -80ºC.

5.4.3. Urinary albumin excretion (UAE)

For each individual we collected two consecutives 24 hour samples spaced no longer than one week between them. UAE was assessed by immunonephelometry (Behring Institute)[263] . Urine samples were stored at 4 ºC in glass tubes and they were processed within the following next 7 days. We considerer as real value the mean of UAE of the two samples. If the difference between the values for the two samples was greater than 25% or if the highest value of creatinine excretion was below than the expected value according to age and body surface area (BSA), then a third sample was picked up.

5.5. Genotyping procedure

DNA was extracted of peripheral leukocytes using Chemagic System (Chemagen, Arnold-sommerfeld-ring2, 52499, Baesweiler, Germany). The extracted DNA met the general requirements:

- Double stranded
- Free of PCR inhibitors
- Not contaminated with other human DNAs or DNA from other organisms
- Not highly degradated

Steps:

1- Reagent preparation and storage

2- Restriction enzyme digestion

3- Ligation

4- PCR

5- PCR purification and elution

6- Quantification of purified PCR product

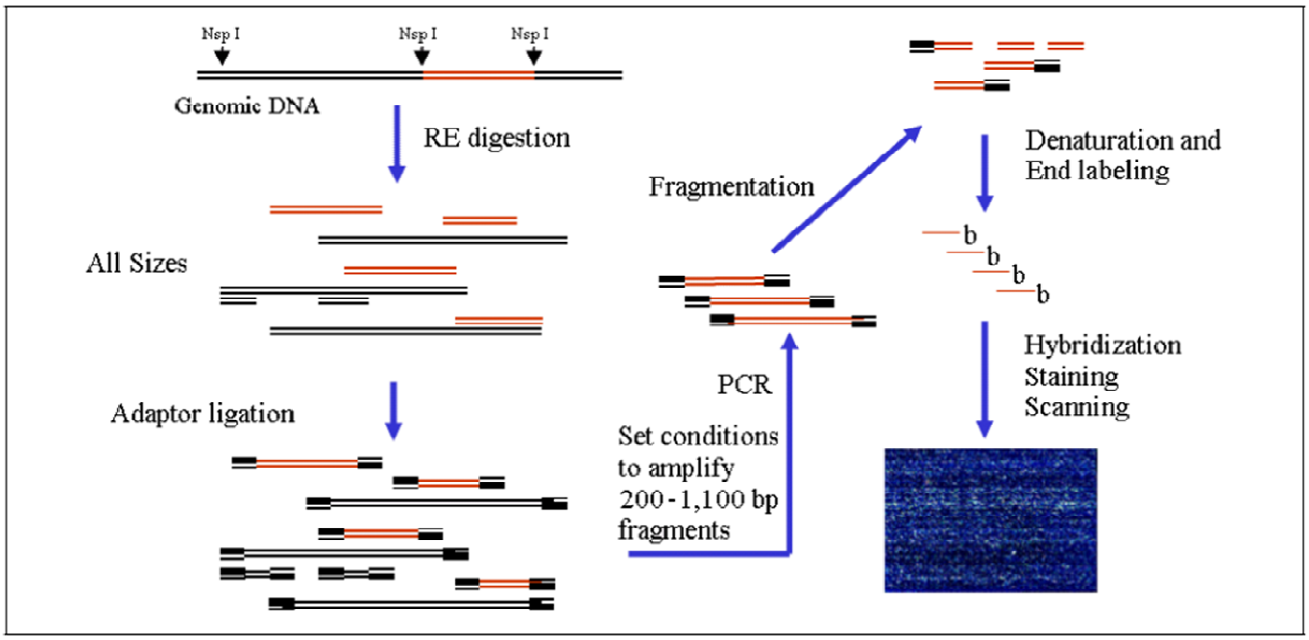
7- Fragmentation

8- Labelling

9- Target hybridization

A resume of this steps for the GeneChip® Mapping Assay - Nsp I is shown in figure 5.1.

Figure 5. 1. *Outline of the assay steps for the mapping assay Nsp-I*



5.5.1. Reagent preparation and storage

Before to start, all the pipettes were calibrated to ± 5%. DNA sample was prepared by: determining the concentration of each sample, diluting each sample to 50 ng/µL using reduced EDTA TE buffer and Aliquoting 5 µL of each sample to a 96-well plate (one sample per well). AccuGENE® water was used as molecular biology-grade water. All reagents (except enzymes) were kept on ice in a cooling chamber that was chilled to 4ºC. Enzymes were stored at -20ºC until they were added to the master mixes. When enzymes were removed from the freeze, they were kept in a cooling chamber that was chilled to -20ºC and placed in ice. All the reagents used for the restriction digestion, ligation and PCR steps were stored in a Pre-PCR clean area. The final DNA concentration was 250 ng (5 µL, each µL contained 50 ng of DNA).

5.5.2. Restriction enzyme digestion

The restriction enzyme used was the Nsp I. The reagents used were: 1 vial BSA (100X; 10 mg/mL), 1 vial NE Buffer 2, 1 vial Nsp I (10 U/µL; NEB) and 2.0 mL of AccuGENE® Water. In this step the reference Genomic DNA 103 (Nsp 100 Rxn Kit P/N 900753) was used as a positive control. The digestion master mix was prepared as follow:

To the 2.0 mL Eppendorf tube the appropriate volumes of AccuGENE® Water, NE Buffer and BSA were added based on figure 5.2.

Figure 5. 2. *Nsp I digestion master mix.*

Reagent	1 Sample	96 Samples (15% extra)
AccuGENE® Water	11.55 µL	1275.1 µL
NE Buffer 2 (10X)	2 µL	220.8 µL
BSA (100X; 10 mg/mL)	0.2 µL	22.1 µL
Nsp I (10 U/µL)	1 µL	110.4 µL
Total	**14.75 µL**	**1628.4 µL**

The Nsp enzyme was removed from the freeze and immediately was placed in a cooler. The enzyme was spinned for 3 seconds before added to the master mix. After that, the master mix was vortexed three times, one second each time. It was spinned for three second and it was placed in the cooling chamber. 135 µL of Digestion Master Mix were aliquoted to each tube of the strip tubes labelled *Dig*. 14.75 µL of Digestion Master Mix were added to each DNA sample in the cooling chamber on ice. The total volume in each well must be 19.75 µL.

Figure 5. 3. *Total volume for each well.*

Reagent	Volume/Sample
Genomic DNA (50 ng/µL)	5 µL
Digestion Master Mix	14.75 µL
Total Volume	19.75 µL

After that the plate was sealed with adhesive film. The centre of the plate was vortexed for 3 second and spinned down at 2000 rpm 30 seconds. Then the plate was loaded into the thermal cycler and the digestion program was run (Figure 5.4).

Figure 5. 4. *500K digest program.*

500K Digest Program	
Temperature	Time
37°C	120 minutes
65°C	20 minutes
4°C	Hold

When the program was finished it was spinned down again at 2000 rpm during 30 seconds before to the next step.

5.5.3. Ligation

Firstly, the following reagents were thawed: adaptor Nsp I and T4 DNA Ligase Buffer (10X) on ice during 20 minutes. Then the ligation master mix was prepared as follow:

To the 2.0 mL Eppendorf tube, the appropriate volumes of the following reagents were added: adaptor Nsp I and T4 DNA Ligase Buffer (10X), based on figure 5.5.

Figure 5. 5. *Nsp I ligation master ix.*

Reagent	1 Sample	96 Samples (15% extra)
Adaptor Nsp I (50 µM)	0.75 µL	82.8 µL
T4 DNA Ligase Buffer (10X)	2.5 µL	276 µL
T4 DNA Ligase (400 U/µL)	2 µL	220.8 µL
Total	**5.25 µL**	**579.6 µL**

After removed the T4 DNA ligase, it was immediately placed in a cooling chamber on ice. Before add to the mater mix it was pulse spinned for 3 second. After the addition of the T4 DNA ligase to the master mix, it was vortexed at high speed 3 times, 1 second each time and then pulse spinned 3 seconds. Then the master mix was placed on ice before to add to the reactions. 48 µL of Ligation Master Mix were aliquoted to each tube of the strip tubes on ice. Then 5.25 µL of Ligation Master Mix were added to each reaction on the Digestion Stage Plate. The volumes are shown in figure 5.6.

Figure 5. 6. *Resume of the volumes.*

Reagent	Volume/Sample
Digested DNA	19.75 µL
Ligation Master Mix*	5.25 µL
Total	**25 µL**

* Contains ATP and DTT. Keep on ice.

Then the plate was tightly sealed, the centre of the plate was vortexed for 3 seconds and was spinned down at 2000 rpm during 30 seconds. The plate was loaded into the thermal cycle and the ligate program was run (Figure 5.7).

Figure 5. 7. *500K ligate program.*

500K Ligate Program	
Temperature	**Time**
16°C	180 minutes
70°C	20 minutes
4°C	Hold

After that, the samples were diluted using the AccuGENE® Water which was placed on ice 20 minutes prior to use it. When the plate was removed from the thermal cycler it was spinned down at 2000 rpm 30 seconds and was placed on the cooling chamber. To make the dilution 10 ml of AccuGENE® Water were poured into the solution basin. 75 µL of the water were added to each reaction. The total volume was 100 µL (Figure 5.8).

Figure 5. 8. *Resume of the volumes.*

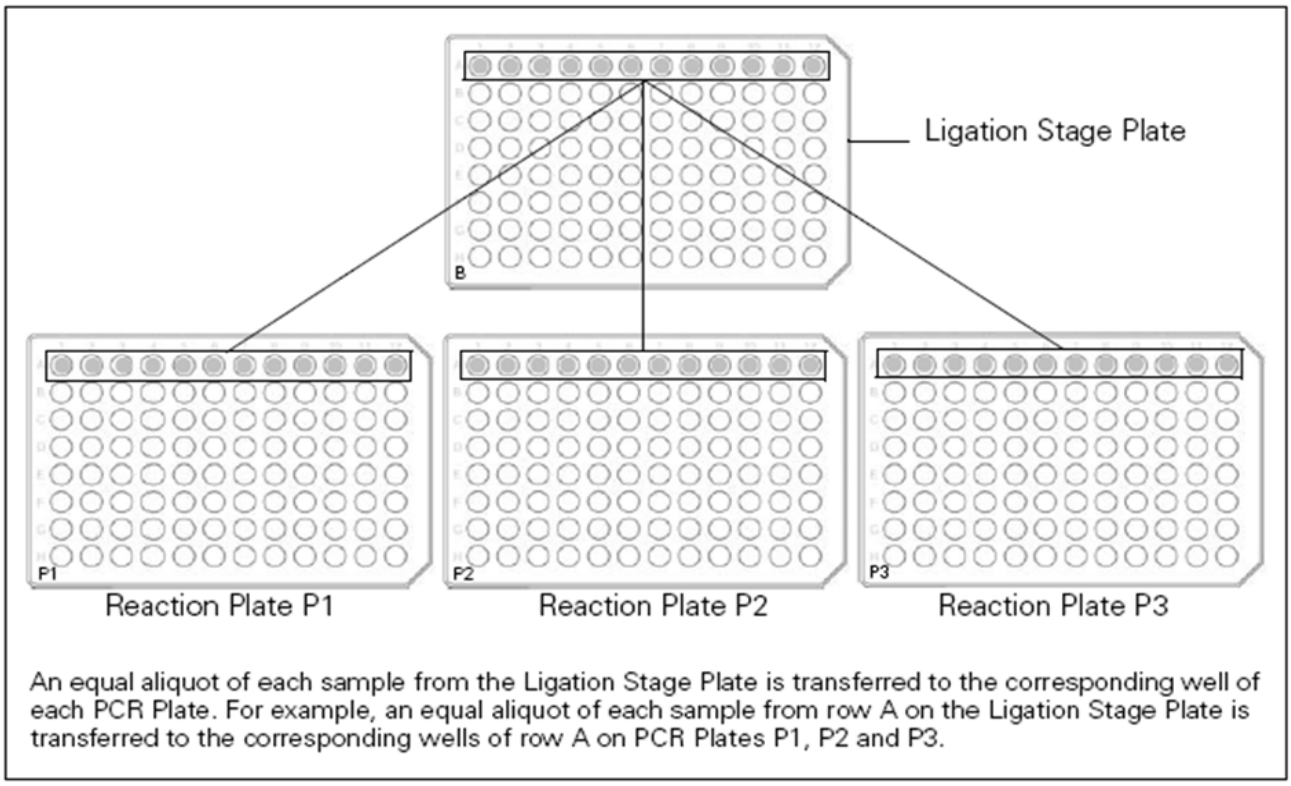
Reagent	Volume/Sample
Ligated DNA	25 µL
AccuGENE® water	75 µL
Total	**100 µL**

Again the plate was tightly sealed with adhesive film, vortexed during 3 seconds, and spinned down at 2000 rpm for 30 seconds. The plate was stored in a cooling chamber on ice prior to the next step.

5.5.4. PCR

Firstly, the following reagents were thawed on ice: TITANIUM *Taq* PCR Buffer, dNTPs, PCR Primer 002. Then 10 µL of sample from each well of the ligation plate were transferred to the corresponded well of the reaction plate according to figure 5.9.

Figure 5. 9. *Transferring equal aliquots of diluted, ligated samples to three reaction plates.*



Then the plates were sealed with adhesive film and placed in cooling chambers on ice. To prepare the PCR master mix the following steps were performed:

To the 50 ml Falcon tube in the cooling chamber the reagents were added in order: The TITANIUM *Taq* DNA polymerase was removed from the freeze and immediately placed in a cooler. The Taq DNA polymerase was pulse spinned for 3 seconds before to add it to the master mix. The master mix was vortexed at high speed three times, 1 second each time. The mix was poured on the solution basin and placed on ice. The composition of the PCR master mix is shown in figure 5.10.

Figure 5. 10. *PCR master mix.*

Reagent	For 1 Reaction	For 3 PCR Plates (15% extra)
AccuGENE® water	39.5 µL	13.082 mL
TITANIUM *Taq* PCR Buffer (10X)	10 µL	3.312 mL
GC-Melt (5M)	20 µL	6.624 mL
dNTP (2.5 mM each)	14 µL	4.637 mL
PCR Primer 002 (100 µM)	4.5 µL	1.490 mL
TITANIUM *Taq* DNA Polymerase (50X)	2 µL	0.663 mL
Total	**90 µL**	**29.808 mL**

Then in the PCR staging area, 90 µL of the PCR master mix were added to each sample to a final volume of 100 µL. The reaction plates were tightly sealed with adhesive film and the centre of each reaction plate was vortexed at high speed for 3 seconds. Then, the plates were spinned down at 2000 rpm during 30 seconds and were kept in cooling chambers on ice until placed into the thermal cycler. After loaded the plate in the thermal cycler the PCR program was run according to figure 5.11.

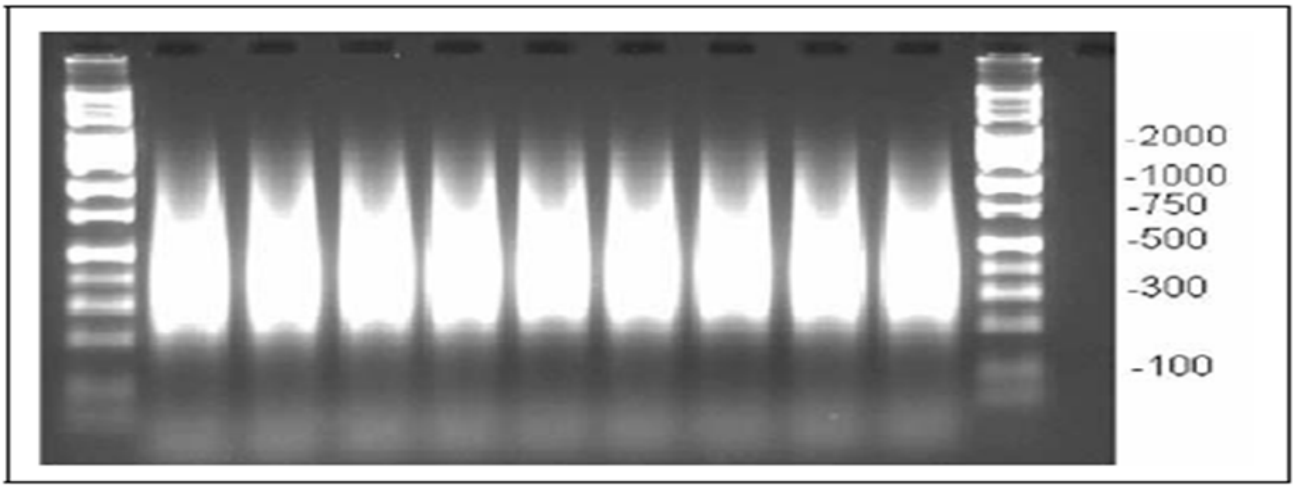
Figure 5. 11. *500K PCR thermal cycler program for the GeneAmp® PCR System 9700.*

500K PCR Program for GeneAmp® PCR System 9700		
Temperature	**Time**	**Cycles**
94°C	3 minutes	1X
94°C	30 sec	} 30X
60°C	45 sec	
68°C	15 sec	
68°C	7 minutes	1X
4°C	HOLD (Can be held overnight)	
Volume: 100 µL		
Specify *Maximum* mode.		

The gels were run to ensure the consistency of the results. After the plates were removed from the thermal cycler, they were spinned down at 2000 rpm during 30 seconds. The plates were placed in the cooling chamber on ice. Then 3 µL of 2X Gel Loading Dye were aliquoted to each well of the three gel plates and 3 µL of each PCR product were transferred into the corresponded well of each gel plates. The plates were sealed with adhesive film and the centre was vortexed and spinned

down at 2000 rpm during 30 seconds. The 6 µL of each plate were loaded onto 2% TBE gels. The gels were run at 120V for 40 minutes to 1 hour to verify that the lengths of the fragments were between 250 bp and 1100 bp. Figure 5.12 shows a correct distribution of the fragments.

Figure 5. 12. *Example of PCR products runs on 2% TBE agarose gel at 120V for 1 hour.*



5.5.5. PCR product purification and elution

Firstly the PCR products plate was thawed to a room temperature and the centre of each plate was vortexed during 3 seconds and spinned down at 2000 rpm during 30 seconds. The EDTA solution was prepared to achieve a final concentration of 0.1 M.

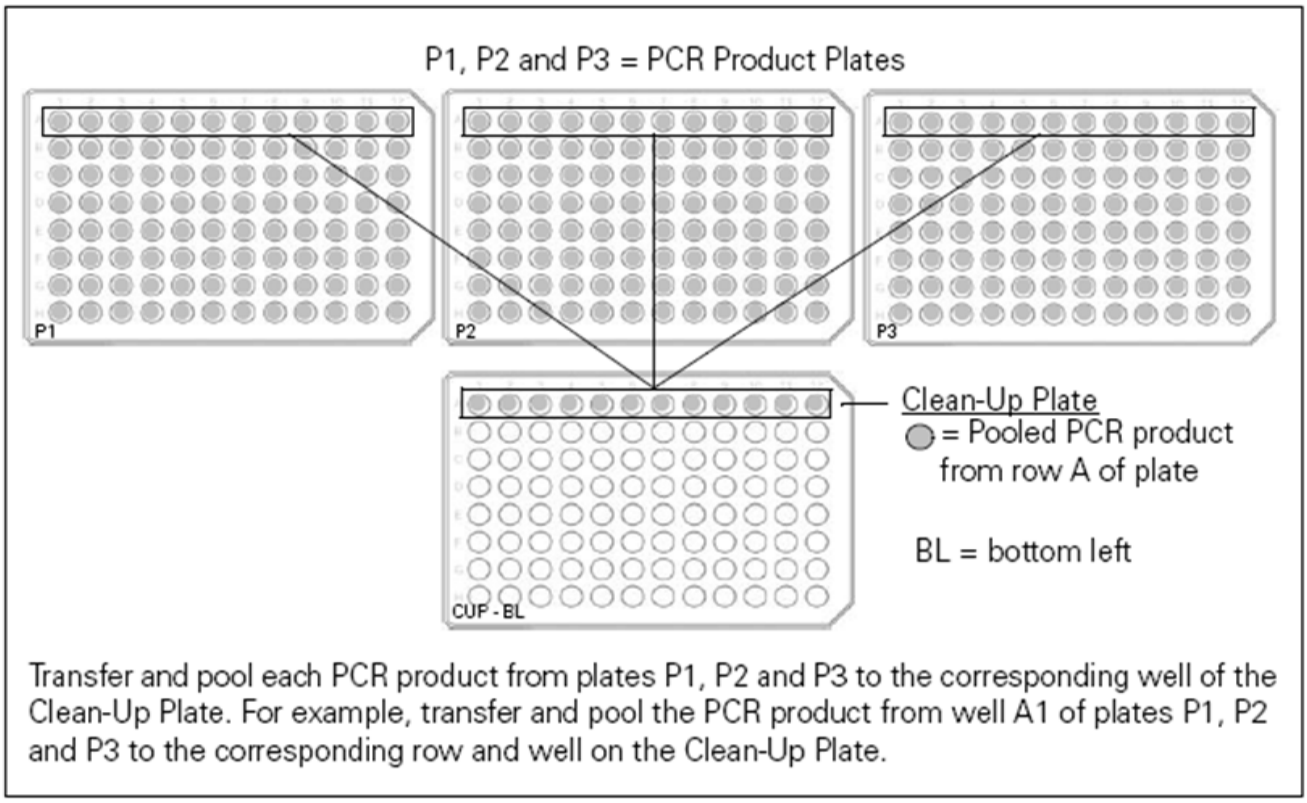
The manifold and collector were connected to a suitable vacuum source able to maintain 600 mbar. The waste tray was placed in the base of the manifold.

3 µL of the diluted EDTA were added to the solution basin. 8 µL of the diluted EDTA were added to each well of the PCR products plate. Then the plate was tightly sealed and vortexed at high speed for 3 seconds and spinned down at 2000 rpm during 30 seconds. Then the plate was putted in a plate holder.

The next step was to prepare the clean-up plate which was labelled to indicate its orientation.

Then the samples were transferred and pooled from the same row and well of each PCR product plate to the corresponding row and well of the Clean-Up Plate. This step is represented in figure 5.13.

Figure 5. 13. *Pooling PCR products onto the Clean-Up Plate.*



The pipette tips were change between each of the three pooled samples. After the procedure it was checked that each well of the PCR product plate was empty. The final volume of the clean-up plate was around 320 µL.

The next step was to purify the PCR products. For this, the clean-up plate with the samples was loaded onto the manifold. To protect the samples of environmental contaminants, the clean-up plate with the samples was covered with the lid of a pipette tips box. The vacuum was turned on and brought up slowly to 600 mbar. The vacuum was checked by trying to lid the middle section of the manifold off the base. It took between 1.5 and 2 hour until all of the wells were dried. Then, the PCR products were washed three times. For this 75 mL AccuGENE® Water were added to a solution basin and 50 µL of water were added to each well. Then the wells were dried during 15-20 minutes. This procedure as a mentioned before was performed three times. After the third wash the manifold was tapped firmly on the bench to get the drops from the side walls went to the bottom. The samples were dried completely for about 45 to 60 minutes and it was checked that the top and the bottom rows were completely dry.

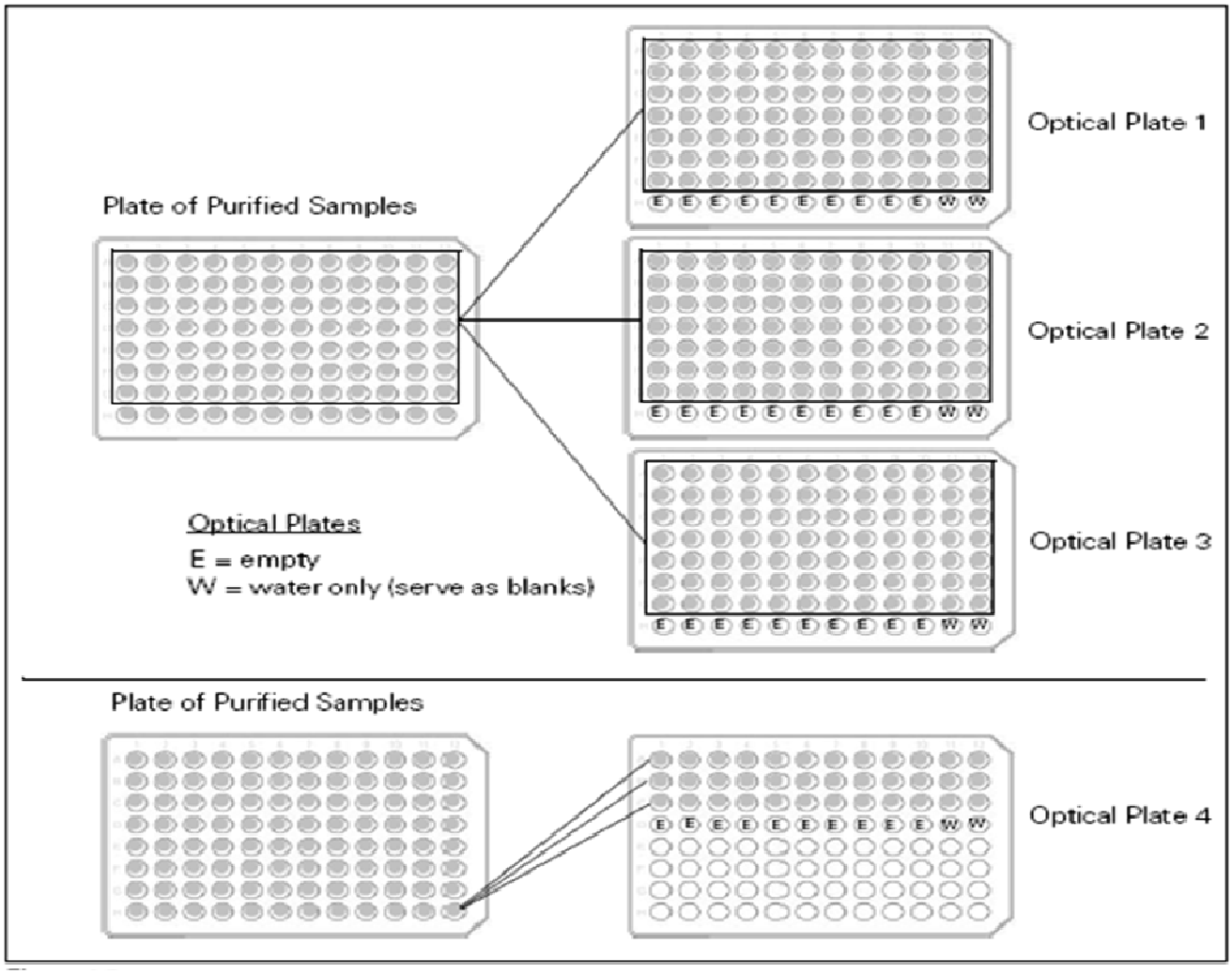
The following step was to elute the PCR products. After turned off the vacuum, the plate was removed from the manifold and then the bottom of the plate was blotted on a thick stack of clean absorbent paper to remove any remaining liquid. Then the bottom of each well was dried with an absorbent wipe. Five millilitres of the RB buffer were aliquoted to the solution basin. 45 µL RB

buffer were added to each well of the plate. The plate was tightly sealed and was shaken at 1000 rpm for 10 minutes at room temperature. Forty five microlitres were transferred of the eluted sample to the correspondent well of a fresh 96 well-plate following the user guide recommendations. That is that the plate was tilted 45ºC and it was pipetted up and down 3-4 times before removing and transferring to the fresh 69 well-plate.

5.5.6. Quantitation and normalization

A 96 well-plate fragment was labelled and was placed in a cooling chamber. The optical plates were also labelled: OP1, OP2, OP3 and OP4. The RB buffer and the eluted PCR products plate were vortexed at high speed for 3 seconds and spinned down at 2000 rpm for 30 seconds. Then, three diluted aliquots of the purified samples were prepared. Seventy five millilitres of room temperature AccuGENE® water were poured into the solution basin. One hundred ninety eight microlitres of water were aliquoted to each well of OP1, OP2, and OP3 and the first four rows of OP4. After that, 2 µL of each purified PCR product were transferred from the purified sample plate to the corresponded well of optical plates. This transference is shown in figure 5.14.

Figure 5. 14. *Loading optical plates with purified sample.*



To ensure that all product was dispensed it was pippeted several times and 2 µL of each purified PCR product were transferred from row H to the correspondent well of the OP4.

A tend fold dilution was obtained as a result. The well containing only water was used as a blank. Then the samples were mixed with a pipette set to 180 µL by pipetting up and down. Then three plates of diluted PCR products were prepared to test.

Next step was to quantify the PCR products with a UV spectrophotometer plate reader. For this, the OD at 260, 280 and 320 nm was measured. Three OD readings were taken for each sample and the average of the three readings was calculated. The undiluted sample concentration was calculated with the following formula:

Sample concentration in µg/µL = Average Sample OD x (0.05 µg/µL) x 100.

The average OD was between 0.5-0.7. The final PCR product concentration was of 2.5 to 3.5 µg/µL and the ratio OD280/OD260 was between 1.8 and 2.0 for the majority of the samples. The OD320 was around zero.

The volume of RB Buffer required to normalize the samples was calculated according to the following formula:

X µL RB Buffer = 45 µL – (Y µL purified PCR product)

Where:

Y = the volume of purified PCR product that contains 90 µg

The value of Y is calculated as:

Y µL purified PCR product = (90 µg) / (Z µg/µL)

Z = the concentration of purified PCR product in µg/µL

The calculated volume of the RB buffer was added to each well of a new 96-well reaction plate. Then the calculated volume (Y) of the purified PCR product was added to the correspondent well with RB buffer. The final volume of each well was 45 µL containing 90 µg of purified PCR product. The plate was sealed with adhesive film and the centre of the plate was vortexed for 3 seconds, spinned down at 2000 rpm for 30 seconds and placed in a cooling chamber.

5.5.7. Fragmentation

Firstly, 50 µL of 10X Fragmentation Buffer were aliquoted to each tube of the 12-tube labelled buffer. Immediately 5 µL of Fragmentation Buffer were added to each sample of the 96-well reaction plate to a final volume of 50 µL. The fragmentation reagent was diluted according to figure 5.15 to a final concentration of 0.05 U/µL.

Figure 5. 15. *Dilution recipes for fragmentation reagent concentrations of 2 and 3 U/µL.*

Reagent	Fragmentation Reagent Concentration	
	2 U/µL	3 U/µL
AccuGENE® water	525 µL	530 µL
Fragmentation Buffer	60 µL	60 µL
Fragmentation Reagent	15 µL	10 µL
Total (enough for 96 samples)	**600 µL**	**600 µL**

For this step the AccuGENE® water and the fragmentation Buffer were added to a 1.5 mL Eppendorf tube on ice and were let to cool on ice. After that the fragmentation reagent was removed from the freeze and was spinned for 3 seconds and placed in a cooler. Then it was added to the 1.5 mL Eppendorf tube. This tube with the diluted fragmentation reagent was vortexed at high speed three times, 1 second each time and spinned 3 seconds. Immediately this diluted fragmentation reagent was added to the samples. For this step 50 µL of diluted Fragmentation

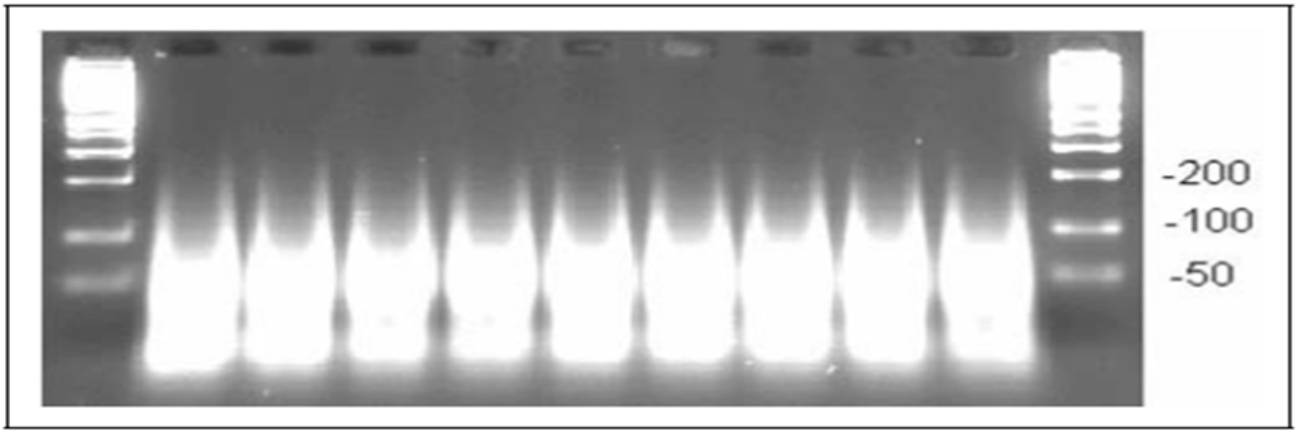
Reagent were aliquoted to each tube of the 12-tube strip labelled FR. From this 5 µL of diluted Fragmentation Reagent were added to each sample to a final volume of 55 µL. The plate was sealed with adhesive film, the centre of the plate was vortexed for 3 seconds and then was placed in a chilled plastic plate holder to spin it down at 4 °C at 2000 rpm for 30 sec. After that the plate was loaded into the thermal cycler and the fragmentation program was run (Figure 5.16).

Figure 5. 16. *500K fragment program.*

500K Fragment Program	
Temperature	**Time**
37°C	35 minutes
95°C	15 minutes
4°C	Hold

To check that the fragmentation was right, 4 µL of each fragmented PCR product were used. This volume was diluted with 4 µL gel loading dye. It was run on 4% TBE gel with the BioNexus All Purpose Hi-Lo ladder at 120V for 30 minutes to 1 hour. The gel was checked according to figure 5.17.

Figure 5. 17. *Typical example of fragmented PCR products runs on 4% TBE agarose gel at 120V.*



5.5.8. Labelling

After thawed the reagents on ice, the labelling master mix was prepared. For this, the volumes of figure 5.18 were added to a 15 mL centrifuge tube on ice.

Figure 5. 18. *Labelling master mix.*

Reagent	1 Sample	96 Samples (15% extra)
TdT Buffer (5X)	14 µL	1545.6 µL
GeneChip® DNA Labeling Reagent (30 mM)	2 µL	220.8 µL
TdT enzyme (30 U/µL)	3.5 µL	386.4 µL
Total	19.5 µL	2152.8 µL

The TdT enzyme was removed from the freeze and immediately placed in the cooler. The enzyme was spinned for 3 seconds before to add it to the master mix. This master mix was vortexed at high speed for 3 seconds and spinned another 3 seconds. To add the labelling master mix to the samples the following steps were done. One hundred seventy eight microlitres of the labelling master mix were aliquoted to each tube of the strip tubes. Then, 19.5 µL of the labelling master mix were aliquoted to each sample to a final volume of 70 µL. To ensure that all the mix was added to our samples, it was pipetted up and down several times. The plate was tightly sealed. The centre of the plate was vortexed at high speed for 3 seconds and spinned down at 2000 rpm during 30 seconds. Then the plate was loaded into the thermal cycle to run the label program (Figure 5.19).

Figure 5. 19. *500K label program.*

500K Label Program	
Temperature	Time
37°C	4 hours
95°C	15 minutes
4°C	Hold
Samples can remain at 4 °C overnight.	

After removed from the thermal cycle it was spinned down at 2000 rpm during 30 seconds.

5.5.9. Target Hybridization

Firstly, the oven was preheated at 49 ºC before to load the samples. Then the hybridation master mix was prepared as follow. To the 50 ml centrifuge tube the reagents were added according to figure 5.20.

Figure 5. 20. *Hybridization master mix.*

Reagent	1 Array	96 Arrays (15% extra)
MES (12X; 1.25 M)	12 µL	1320 µL
Denhardt's Solution (50X)	13 µL	1430 µL
EDTA (0.5 M)	3 µL	330 µL
HSDNA (10 mg/mL)	3 µL	330 µL
OCR, 0100	2 µL	220 µL
Human Cot-1 DNA® (1 mg/mL)	3 µL	330 µL
Tween-20 (3%)	1 µL	110 µL
DMSO (100%)	13 µL	1430 µL
TMACL (5 M)	140 µL	1540 mL
Total	**190 µL**	**20.9 mL**

It was ensured that all the components mixed well. Then 20.9 mL of the Hybridization Master Mix were poured into a solution basin. Then 190 µL of Hybridization Master Mix were added to each sample of the label plate to a final volume of 260 µL. Then the plate was tightly sealed and vortexed for 3 minutes. The plate was cut into 4 strips and each strip of 24 samples was putted into a plate holder. This was spinned down at 2000 rpm for 30 seconds and was placed into the thermal cycler to run the Hybridation program (Figure 5.21).

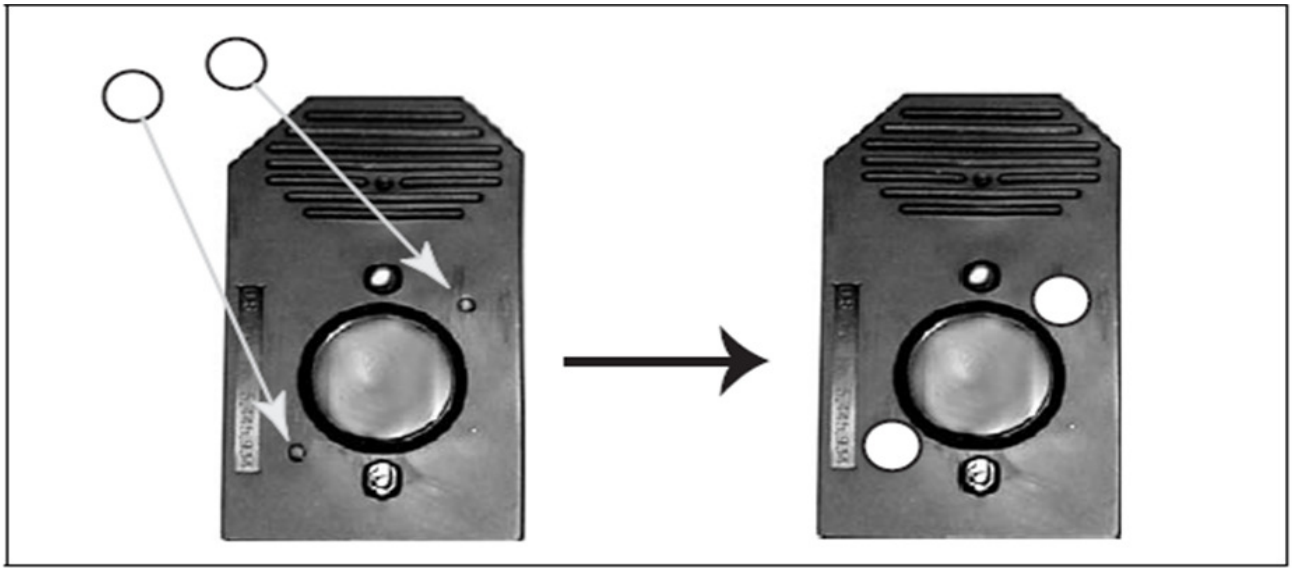
Figure 5. 21. *500K Hyb program.*

500K Hyb Program	
Temperature	Time
95°C	10 minutes
49°C	Hold

Each set of 24 wells was placed separately. The set which was not in the thermal cycler was placed into the cooling chamber on ice.

Next step was loaded the sample onto arrays. When the plate was at 49ºC, it was removed from the thermal cycler. Then 200 µL of denatured sample were pipetted from each well and were injected into the array one by one. Another operator was ready to cover the septa on each array with a Tough-Spot according to figure 5.22.

Figure 5. 22. *Applying Tough-Spots® to the array cartridge.*



When 4 arrays were loaded, the septa were covered and loaded into an oven tray evenly spaced. Immediately the tray was placed into the oven and it was ensured that they rotated at 60 rpm at all times. The arrays were let to rotate at 49 ℃, 60 rpm for 16 to 18 hours.

5.5.10. Washing, staining, and scanning Arrays

Firstly the reagents for this stage were prepared according to tables 5.1-5.3.

Table 5. 1. *Non stringent and stringent wash buffer.*

Wash A: Non-Stringent Wash Buffer	**Wash B: Stringent Wash Buffer**
(6X SSPE, 0.01% Tween 20)	(0.6X SSPE, 0.01% Tween 20)
For 1000 mL:	For 1000 mL:
300 mL of 20X SSPE	30 mL of 20X SSPE
1.0 mL of 10% Tween-20	1.0 mL of 10% Tween-20
699 mL of water	969 mL of water
Filter through a 0.2 µm filter	Filter through a 0.2 µm filter
Store at room temperature	Store at room temperature
	The pH should be 8

Table 5. 2. *Anti-streptavidin antibody.*

0.5 mg/mL Anti-Streptavidin Antibody
Resuspend 0.5 mg in 1 mL of water
Store at 4℃

Table 5. 3. *MES stock buffer and array holding buffer.*

12X MES Stock Buffer	1X Array Holding Buffer
(1.25 M MES, 0.89 M [Na+])	(Final 1X concentration is 100 mM MES, 1M [Na+], 0.01% Tween-20)
For 1,000 mL:	For 100 mL:
70.4g of MES hydrate	8.3 mL of 12X MES Stock Buffer
193.3g of MES Sodium Salt	18.5 mL of 5 M NaCl
800 mL of Molecular Biology Grade Water	0.1 mL of 10% Tween-20
Mix and adjust volume to 1,000 mL	73.1 mL of water
The pH should be between 6.5 and 6.7	Store at 2℃ to 8℃, and shield from light
Filter through a 0.2 µm filter	

At this time, a new experiment in the GeneChip® Operating Software (GCOS) 1.4 client was opened

The Fluidics Station 450 was used to wash and stain the probe arrays and GCOS was use to operate it. To prime the fluidics station, Prime_450 was chosen in the protocol drop-down list.

The intake buffer reservoir A was changed to Non-Stringent Wash Buffer, and intake buffer reservoir B to Stringent Wash Buffer. The priming was done according to the instructions on the screen.

The following procedures were done to wash and stain the probe array. After 16-18 hours of hybridation, the hybridation cocktail was removed from the probe array and set it aside in a micro centrifuge vial. Then the probe array was filled completely with 270 µL of Array Holding Buffer.

The staining reagents were prepared according to figure 5.23-5.26.

Figure 5. 23. *Stain buffer.*

Components	1X	Final Concentration
H_2O	800.04 µL	
SSPE (20X)	360 µL	6X
Tween-20 (3%)	3.96 µL	0.01%
Denhardt's (50X)	24 µL	1X
Subtotal	1188 µL	
Subtotal/2	594 µL	

Figure 5. 24. *SAPE solution mix.*

Components	Volume	Final Concentration
Stain Buffer	594 µL	1X
1 mg/mL Streptavidin Phycoerythrin (SAPE)	6.0 µL	10 µg/mL
Total	600 µL	

Figure 5. 25. Antibody solution mix.

Components	Volume	Final Concentration
Stain Buffer	594 µL	1X
0.5 mg/mL biotinylated antibody	6 µL	5 µg/mL
Total	600 µL	

Figure 5. 26. *Array holding buffer.*

Components	Volume
MES Stock Buffer (12X)	8.3 mL
5 M NaCl	18.5 mL
Tween-20 (10%)	0.1 mL
Water	73.1 mL
Total	100 mL

Then, 820 µL of Array Holding Buffer were added to each micro centrifuge tube.

Figure 5. 27. *FS-450 fluidics protocol - Antibody amplification for mapping targets.*

	49 Format (Standard) Mapping 500Kv1_450
Post Hyb Wash #1	6 cycles of 5 mixes/cycle with Wash Buffer A at 25°C
Post Hyb Wash #2	24 cycles of 5 mixes/cycle with Wash Buffer B at 45°C
Stain	Stain the probe array for 10 minutes in SAPE solution at 25°C
Post Stain Wash	6 cycles of 5 mixes/cycle with Wash Buffer A at 25°C
2nd Stain	Stain the probe array for 10 minutes in Antibody Stain Solution at 25°C
3rd Stain	Stain the probe array for 10 minutes in SAPE solution at 25°C
Final Wash	10 cycles of 6 mixes/cycle with Wash Buffer A at 30°C. The final holding temperature is 25°C
Filling Array	Fill the array with Array Holding Buffer.

Wash Buffer A = non-stringent wash buffer
Wash Buffer B = stringent wash buffer

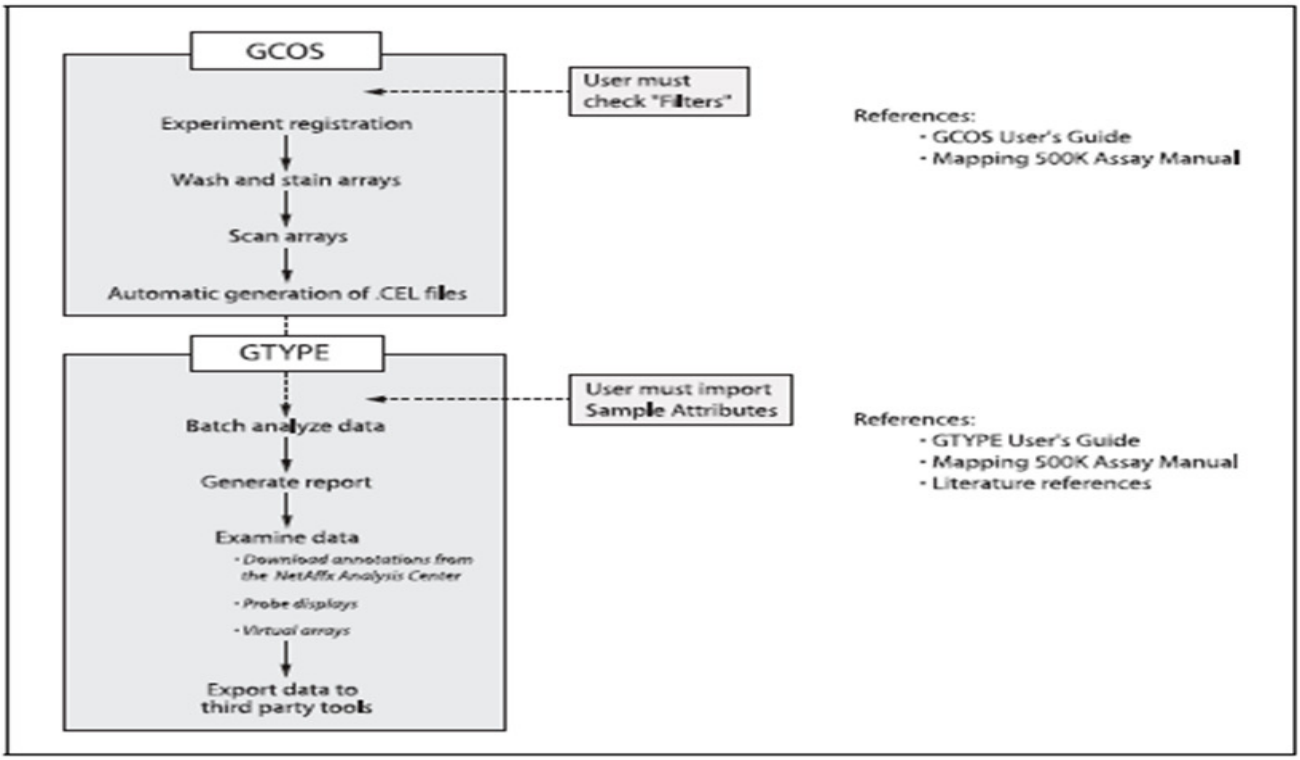
Instructions of the Fluidics Station 450 were followed to wash and stain the probe array. After staining was finished, the micro centrifuge vials containing stain were removed and replaced with three empty micro centrifuge vials as prompted. If bubbles were presented, they were removed according to the instructions.

Next step was to scan the probe array with GeneChip® Scanner 3000 7G. If the scan was not performed immediately, the probe arrays were kept at 4ºC in the dark until they were ready for scanning. All the scans were performed within 24 hour. The GeneChip® Scanner 3000 7G was operated by means of the GCOS Software 1.4

Before scanning the probe array cartridge, Tough-Spots®, label spots were applied to each of the two septa on the probe array cartridge to prevent leaking of fluids from the cartridge during scanning. Then it was ensured that the spots did not interfere with the focus.

After the scanning, the analysis workflow was performed using GeneChip® Operating Software (GCOS) 1.4, GeneChip® Genotyping Analysis Software (GTYPE) 4.0 and Genotyping Console 3.0. The analysis workflow is shown in figure 5.28.

Figure 5. 28. *Data analysis workflow for GeneChip® Human Mapping 250K arrays.*



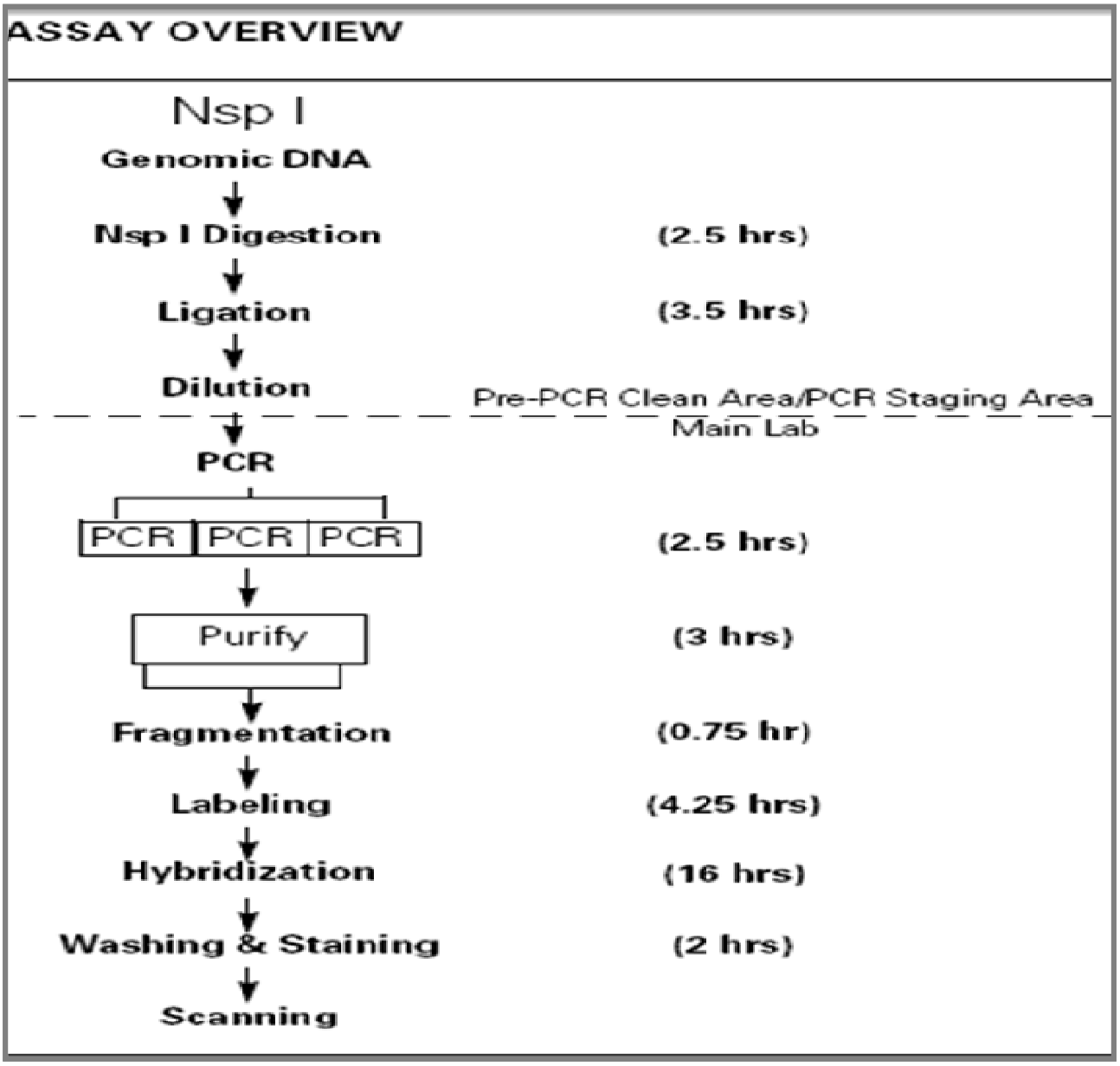
MPAM calling algorithm was used in a subset of SNPs to obtain the MCR and MDR metrics. The relation between the MCR and MDR parameters obtained after genotyping was used to evaluate the possible contamination of the samples. The call rate of the reference genomic DNA 103 sample was used as an indicator that all the steps worked properly. The quality control parameters are shown in figure 5.29.

Figure 5. 29. *Dynamic model mapping algorithm report metrics.*

Metric	Description
Call Rate (SNP Call)	Good first pass evaluation. If the genomic DNA sample is of equivalent quality and purity to the Reference Genomic DNA 103, then the Call Rate should be similar when analyzed at the same algorithm settings.
Reference Genomic DNA 103 Call rate	Process control to show that assay steps are being performed correctly.
MDR – MCR	Difference can be used to identify sample contamination.
Shared SNPs	Evaluate possible sample mix ups.
Oligonucleotide controls	Help evaluate hybridization, fluidics and scanning steps.

To resume all the steps, an overview of the assay is shown in figure 5.30.

Figure 5. 30. *GeneChip® Mapping 500K assay overview.*



5.6. Management of the genotyping microarray data

The intensity files (.CEL) were obtained by the use of the GCOS (Gene-Chip Operating Software). The Affymetrix® Genotyping Console™ 3.0 (AFFYMETRIX, INC. 3420 Central Expressway ,Santa Clara, CA 95051 USA) was used to assessed the quality of the intensity files using the Dynamic Model (DM) algorithm[264].

Those files with a poor quality control (QC) call rates, QC call rate <93%, were not considering for genotyping. The BRLMM algorithm[265] was used to infer the genotypes based on the intensity signal of the two alleles *A* and *B*. We used the Affymetrix power tool (apt) probeset genotype (apt-1.10.2) to convert the CEL files into the genotyping files. As we did not have a great number of subjects we were able to run the BRLMM algorithm for all the samples at the same time. We did not have to divide them into batches of equal or different sizes what can affect the results of the downstream analysis[266].

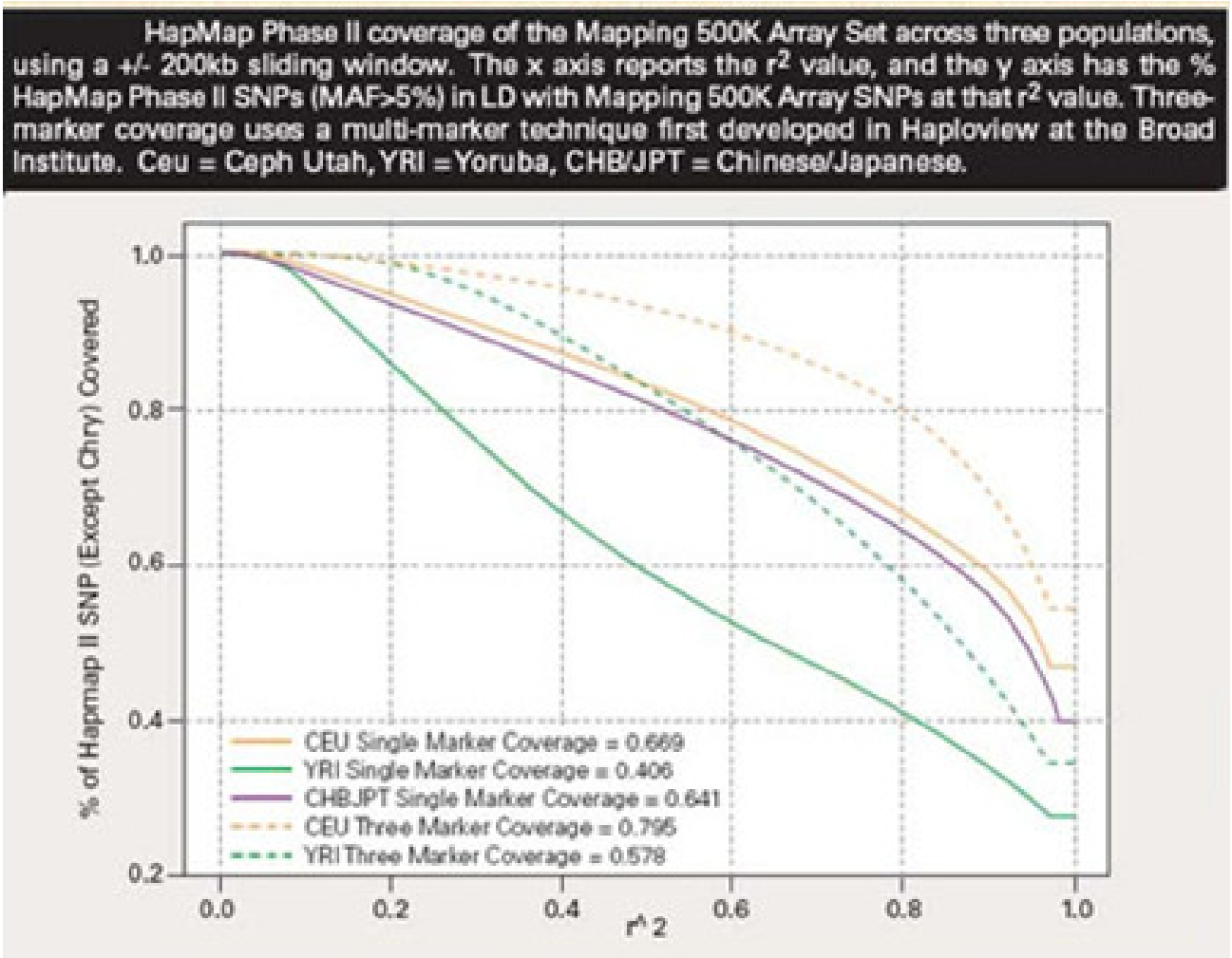
The cluster plots for the main associated SNPs were obtained with the Affymetrix software Genotyping Console version 3.0.1.

5.7. Resume of the genotyping procedure

The DNA was extracted from leukocytes of peripheral blood (5-10 mm). The DNA was purified following standard procedures (Chemagic Magnetic Separation Module I, chemagen Biopolymer-Technologie AG, Arnold-Sommerfeld-Ring 2 D-52499 Baesweiler Germany) [267].

As it had been commented before, we used the Nsp chip of the GeneChip Human Mapping 500K of Affymetrix for the genotyping. This chip includes more than 250000 SNPs across the genome. The high-density chip has a good coverage of the genome and a good coverage between populations (Figure 5.31).

Figure 5. 31. *HapMap Phase II coverage of the Mapping 500K array Set.*



The samples were processed following the quality recommendations of the manufacturer (https://www.affymetrix.com/support/downloads/manuals/500k_assay_manual.pdf).

The Nsp enzyme was used as restriction enzyme and the Nsp I adaptor in the ligation stage. DNA fragments within the range 200-1100 bp were amplified by PCR with only one primer. For the PCR stage we use the DNA Titanium™ Taq polymerase. For the hybridation stage we used the

thermocycler 7900 of Applied Biosystems (Applied Biosystems, 850 Lincoln Centre Drive Foster City, CA 94404, USA). The GeneChip® Scanner 3000 7G was used in the fluorescence analysis. During all the stages suitable quality control procedures were performed to the accuracy of the genotyping[268].

5.8. Statistical analysis

5.8.1. General statistics

Quantitative variables are expressed as mean ± standard deviation (SD). Qualitative variable are expressed as entire number and percentage. Treatment is described as a categorical variable based on the number of drugs which the individual is taking (less than two drugs or more than two drugs). Microalbuminuria and albuminuria will be considered as both qualitative variable (yes or not) and quantitative trait according to the UAE values. To assess the association specifically with microalbuminuria we discharged those subjects with UAE ≥ 300 mg/day.

Binomial multivariate logistic regression will be used to analyze what clinical factors explain the presence of microalbuminuria including interactions between variables. The introduction method will be used to construct the model. Those variables without influence in the presence of microalbuminuria will be deleted according to the score value. The best model with the lowest number of variables will be selected according to the -2log likelihood and R square values and also considering the significance for the Hosmer-Lemeshow test.

In the same way, multiple linear regression will be used to find those clinical co-variables which influence the changes in microalbuminuria as a continuous trait. Again the best model with the lowest number of variables will be selected based on the R square and corrected R square values.

5.8.2. Genetic statistics

5.8.2.1. Individual SNPs association with microalbuminuria and albuminuria (qualitative traits)

The association analysis between the selected polymorphisms and the phenotype will be assessed by comparing the allele frequencies between cases and controls. The basic statistical tests that will be used include: standard allelic test using chi-square test (1df) or the Fisher's exact test.

We will also include the following test to asses the association under different genetic models:

- Cochran-Armitage trend test
- Genotypic (2 df) test
- Dominant gene action (1df) test
- Recessive gene action (1df) test

One advantage of the Cochran-Armitage test is that it does not assume Hardy-Weinberg equilibrium, as the individual, not the allele, is the unit of analysis. The genotypic test provides a general test of association in the 2-by-3 table of disease (Case-Control)-by-genotype. The dominant and recessive models are tests for the minor allele which is the less frequent allele according to the data for each polymorphism. If we considerer D as the minor allele and d as the major allele then we will assess the following comparisons (Table 5.4).

Table 5. 4. *Diagram of the comparison between different genetic models.*

Allelic		D	versus	d	
Dominant		(DD, Dd)	versus	dd	
Recessive		DD	versus	(Dd, dd)	
Genotypic	DD	versus	Dd	versus	dd

For each of the test described above we will obtain the chi-square test, the degree freedom (DF) and the asymptotic p-value. We also performed the adaptive permutations analysis for the main association analysis. In this approach, if after only 10 permutations it is seen that for 9 of these the permuted test statistic for a given SNP is larger than the observed test statistic, there is little point in carrying on, as this SNP is incredibly unlikely to ever achieve a highly significant result. This greatly speeds up the permutation procedure, as most SNPs (that are not highly significant) will drop out quite quickly, making it possible to properly evaluate significance for the handful of SNPs that require millions of permutations. Naturally, the precision with which one has estimated the significance p-value (i.e. relating from the number of permutations performed) will be correlated the significance value itself -- but for most purposes, this is precisely what one wants, as it is of little interest whether a clearly un-associated SNP really has a p-value of 0.78 or 0.87. Permutation procedures provide a computationally intensive approach to generating significance levels empirically. Such values have desirable properties: for example, relaxing assumptions about normality of continuous phenotypes and Hardy-Weinberg equilibrium (HWE), dealing with rare alleles and small sample sizes, providing a framework for correction for multiple testing, and controlling for identified substructure or familial relationships by permuting only within cluster.

5.8.2.2. Individual SNPs association with UAE (quantitative trait)

The association between individual SNPs and the quantitative trait (microalbuminuria mg/24 hours) will be perform using either asymptotic (likelihood ratio test and Wald test) or empirical significance values. With these test we will obtain the following measurements (Table 5.5).

Table 5. 5. *Linear regression parameters.*

BETA	Regression coefficient
SE	Standard error
R2	Regression r-squared
T	Wald test (based on t-distribution)
P	Wald test asymptotic p-value

We will also determinate the means for microalbuminuria for each of the genotypes with the SD as well as the number and frequency of each genotype for all the SNPs.

5.8.2.3. Individual SNPs association with albuminuria, microalbuminuria and UAE (qualitative and quantitative traits) including significant co-variables

Linear and logistic regression models (quantitative and qualitative traits respectively) will be used to include important covariates in the analysis such as age, sex, BMI, SBP, DBP, glucose, eGFR and treatment. We will calculate the Beta regression coefficients and the OR with the correspondent confident intervals, the coefficient for the t-statistic and the asymptotic p-value for that test for all the SNPs.

5.8.2.4. Multiple comparison

The following test will be used to address the multiple comparison problems (Table 5.6)

Table 5. 6. *Test for multiple comparisons.*

BONF	**Bonferroni single-step adjusted p-values**
HOLM	Holm (1979) step-down adjusted p-values
SIDAK_SS	Sidak single-step adjusted p-values
SIDAK_SD	Sidak step-down adjusted p-values
FDR_BH	Benjamini & Hochberg (1995) step-up FDR control
FDR_BY	Benjamini & Yekutieli (2001) step-up FDR control

Although it is the most stringent one, we used the Bonferroni correction to considerer a results as significant. The more stringent a multiple testing correction, the less false positive genes are allowed. The trade-off of a stringent multiple testing correction is that the rate of *false negatives* (genes that are called non-significant when they are) is very high.

5.8.2.5. Haplotype analysis

All tests described above are based on single SNP tests. Haplotypes will be study using the proxy association approach taking into account the main associated SNPs in the individual analysis. Specifically, given a particular (reference) SNP this approach involves a) finding flanking markers and haplotypes (proxies) that are in strong linkage disequilibrium with the reference SNP and, b) testing these proxies for association with disease, within a haplotype-based framework. With this we will obtain the following benefits:

- technical validation of single SNP results (by looking for flanking haplotypes involving different markers that also show the same result).
- refining a single SNP association signal (is there a stronger association with a local haplotype?).
- More robust single SNP tests (by framing single SNP tests within an haplotypic framework, some degree of control against non-random genotyping failure can be achieved).

The R-square and the D´statistic (defined as the linkage disequilibrium measure, D, divided by the theoretical maximum for the observed allele frequencies) will be used to measure the linkage disequilibrium (LD). Haplotypes frequencies will be estimated by the Expectation Maximization Algorithm (EM).
Tag-SNPs, LD and haploblocks were calculated using Haploview version 3.32 [269]. The haploblocks were defined by mean of the confidence intervals (Gabriel et al[270]).

5.8.2.6. Haplotype-based association tests with generalized linear models (GLM)

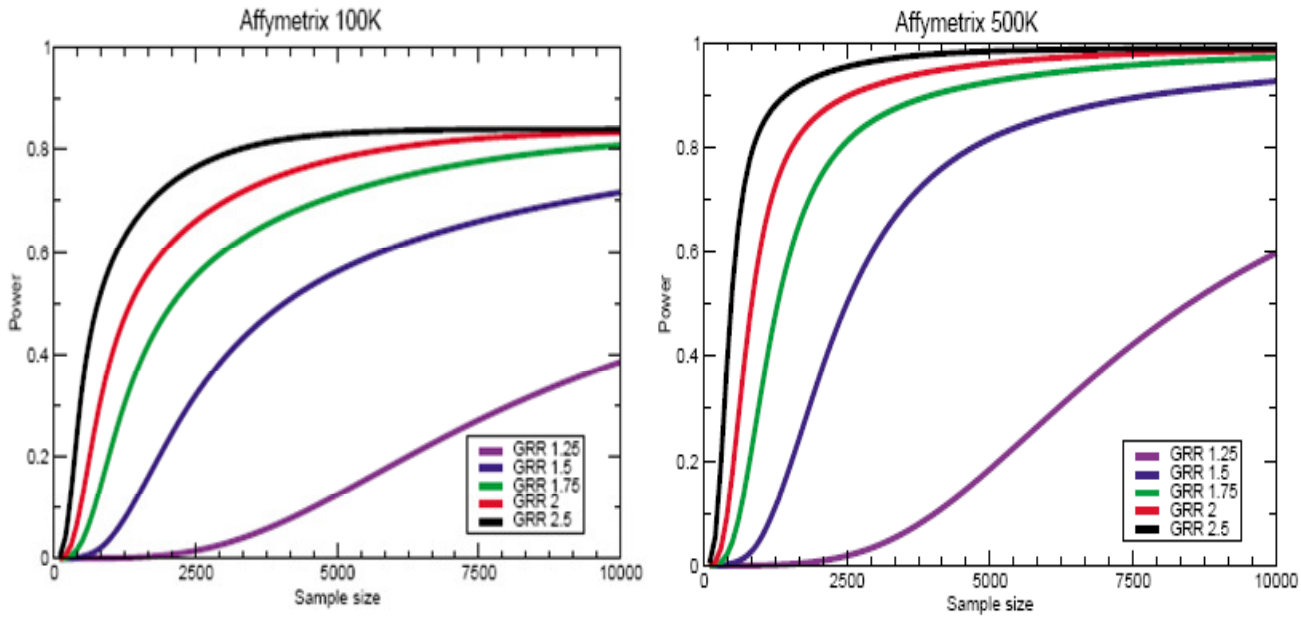
We will also use linear and logistic regression to perform haplotype-based association analysis. These will let to include the main co-variables (Age, sex, BMI, SBP, DBP, glucose and eGFR) into the haplotype association analysis for those SNPs which showed association in the individual analysis with co-variables.
The individual SNP and haplotype analysis in both case-control (qualitative trait: albuminuria or microalbuminuria) and quantitative trait will be performed with the program PLINK v.1.06 developed by Shaun Purcell (http://pngu.mgh.harvard.edu/purcell/plink/)[271]. An extended information about the statistical tests that are used can be found in the web site.

5.8.2.7. Genetic statistical power

To see our statistical power we took into consideration some of the graphs which other authors[272] had developed for GWAS analysis using either Affymetrix or Illumina chips and considering the CEU HapMap population. According to these graphs (Figure 5.32 and 5.33), it is

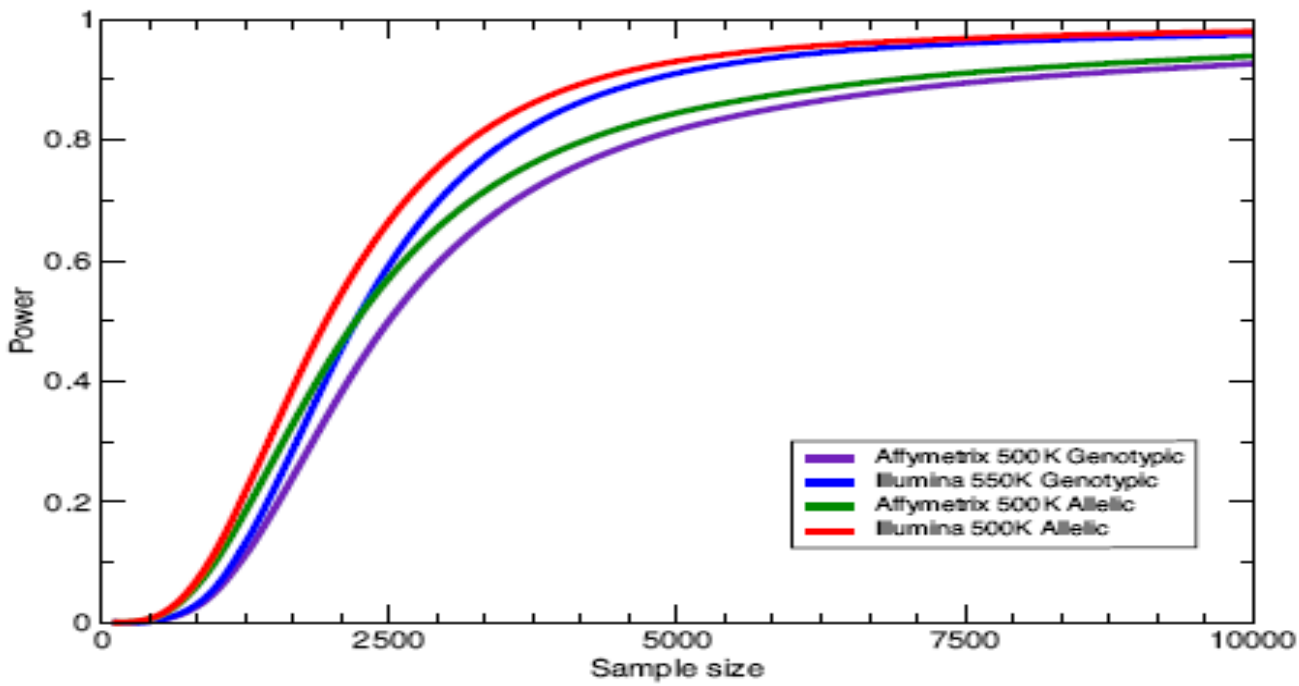
quite clear that our study is underpowered to detect OR equal or below 1.5. These graphs assume an equal number of cases than controls.

Figure 5. 32. *Power for the test of genotypic association as a function of sample size at different genotype relative risks (GRR).*



These figures 5.32 and 5.33 are for the CEU HapMap population when the number of cases equals the number of controls and a multiplicative model is used.

Figure 5. 33. *Power for genotypic and allelic tests.*



Data is shown for a GRR of 1.5 under a multiplicative model, the CEU HapMap population, and the specified genotyping system.

We also used several programs such as the Power for Genetic Association (PGA)[273] software, developed by Menashe and cols and the free software PAWE-3D[274] (http://linkage.rockefeller.edu/pawe3d/) to calculate the statistical power making different assumptions but as I commented before with would need a much bigger sample size to detect OR 1.5 with an statistical power of 0.8.

5.8.2.8. Quantile-Quantile plots

Quantile-Quantile (Q-Q) plots are constructed by ranking a set of values of a statistic from smallest to largest (the 'order statistics') and plotting them against their expected values, given the assumption that the values have been sampled from a distribution of known theoretical form (in our case, the chi-squared distribution, usually on one degree of freedom—for example, the distribution of our trend tests under the null hypothesis).

Deviations from the line of equality indicate either that the theoretical distribution is incorrect, or that the sample is contaminated with values generated in some other manner (for example, by a true association). The Q-Q plots for the p-values obtained in the association analysis were made with the statistical software R version 2.8.1[275].

Results

6. RESULTS

6.1. Clinical characteristics of the study population

The final sample size comprised of 201 controls and 102 cases. Among the cases, 86 (84.3%) had an UAE between 30-299 mg/day and 16 patients (15.7%) had UAE values equal or higher than 300 mg/day. As expected the cases were older than the controls and also had greater values of 24 hour BP, BMI, and fasting glucose. Interestingly there were not differences regarding to the office BP levels. The GFR estimated by MDRD was significantly lower in cases than in controls but not the CC estimated by Cockcroft-Gault formula. Regarding lipids, the LDL was significantly lower in cases than in controls due to the major percentage of individuals under statins treatment. Also the number of lowering blood pressure drugs was significantly higher in cases than it was in controls as well as the percentage of individuals with metabolic syndrome according to the ATPIII criteria[276].

The main characteristics of the study population grouped by the case-control status are shown in table 6.1.

Table 6. 1. *Main characteristics of the study population.*

	NORMOALBUMINURICS N =201	**ALBUMINURICS N=102 Microalbuminurics =86 (84.3%) Macroalbuminurics=16 (15.7%)**
AGE (years)	42.3±10.6	46.3±12.5**
GENDER (Male/Female)	100(49.8)/101(50.2)	60(58.8)/42(41.2)
BMI (Kg/m2)	28.3±4.1	29.7±4.6*
OFFICE SBP	146.6±15.3	147.5±20.3
OFFICE DBP	91.3±10.5	90.6±15.3
24 H SBP	132.5±13.5(n=156)	145.4±15.6**(n=58)
24 H DBP	84.4±9.9(n=156)	93.9±11.2**(n=58)
FASTING GLUCOSE (mg/dl)	100.2±13.9	107.6±17.5**
INSULIN	4.59±6.0(n=32)	6.09±12.2(n=38)
HOMA	1.15±1.5(n=32)	1.93±3.9(n=38)
UREA (mg/dl)	34.2±8.2	42.4±20.9**
CREATININE (mg/dl)	0.94±0.23	1.09±0.51**
GFR (ml/min) (Cockroft-Gault)	104.3±32.5	100.5±36.3
GFR (ml/min) (MDRD)	83.9±24.5	76.9±21.9*
UAE (mg/day)	8.3±5.7	292.3±881.2**
TRIGLYCERIDES (mg/dl)	132.7±74.0	152.8±122.2
TOTAL CHOLESTEROL (mg/dl)	208.0±37.2	204.7±40.5
LDL CHOLESTEROL (mg/dl)	131.6±33.7	114.8±39.2**
HDL CHOLESTEROL (mg/dl)	47.7±11.6	47.9±12.3
LVM (g)	188.4±83.3(n=87)	204.4±109.2(n=36)
ANTYHYPERTENSIVE DRUGS:		
Less than 2 drugs (n/%)	151(96.8)	65 (73)**
More than 2 drugs (n/%)	5 (3.2)	24 (27)**
METABOLIC SYNDROME (n/%)	46 (22.9)	43 (42.2) **

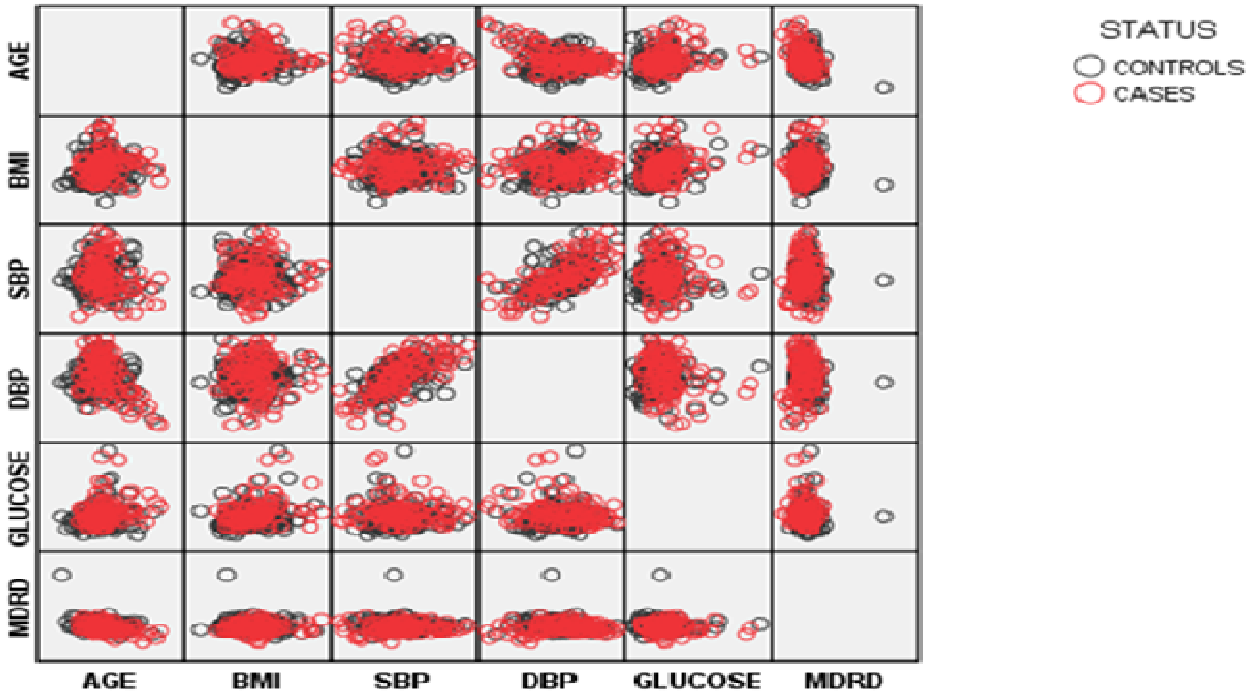
* and ** significant differences between the normoalbuminuric and albuminuric group (* p-value<0.05, **p-value<0.005).

6.2. Clinical factors associated with albuminuria, microalbuminuria and UAE

6.2.1. Logistic regression model for albuminuria and microalbuminuria

To analyze the impact of the clinical factors in the presence of albuminuria we constructed several logistic regression model by using the enter mode. Previously we observed the scatter plot to see the relationship between the different variables included in the analysis.

Figure 6. 1. *Scatter plot for the relationship between pairs of variables.*



In the univariate analysis only age, glucose, eGFR by MDRD and the number of drugs were significantly associated with albuminuria. When including all these variables in the logistic regression (LR) model, only glucose and the numbers of drugs were independently associated with the risk of albuminuria. Those hypertensive patients receiving 2 or more drugs had an increase risk of albuminuria (OR 7.83 (3.61-16.90), p<0.0001). The result of the logistic regression model for the clinical variables is shown in table 6.2

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.**Table 6. 2 .** *Result of the logistic regression for albuminuria.*

		B	S.E.	Wald	df	Sig.	OR	95.0% C.I for OR	
								Lower	Upper
Step 1(a)	AGE (years)	-0.17	0.17	1.100	1	0.294	0.983	0.951	1.015
	SEX (male vs female)	0.79	0.320	0.60	1	0.806	1.082	0.577	2.027
	BMI (Kg/m^2)	-0.10	0.040	0.059	1	0.808	0.990	0.916	1.071
	SBP (mmHg)	.009	.012	0.526	1	.468	1.009	.985	1.034
	DBP(mmHg)	-0.002	.018	0.007	1	.932	0.998	.964	1.034
	GLUCOSE (mg/dl)	.025	.011	4.962	1	**.026**	**1.026**	**1.003**	**1.049**
	eGFR MDRD (mL/min/1.73 m^2)	-0.13	0.008	2.468	1	0.116	0.987	0.972	1.003
	Nº DRUGS (2 or more vs less than 2)	2.058	.394	27.221	1	**.000**	**7.832**	**3.615**	**16.968**
	Constant	-2.920	2.339	1.559	1	.212	.054		

We obtained similar results when we excluded those patients with macroalbuminuria (UAE≥300 mg/day).

6.2.2. Linear regression model for UAE

The study of clinical factors associated with microalbuminuria was also performed by using a multiple regression analysis. Age, sex, BMI, SBP, DBP, glucose, eGFR by MDRD and the number of drugs explained 14% of the variation in the UAE levels (R square 0.140). In this model the most significant variables were the number of drugs (2 or more) and the eGFR. The result of the linear regression model is shown in table 6.3.

Table 6. 3. *Result of the linear regression analysis for UAE.*

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	1021.681	466.608		2.190	.030	102.200	1941.161
	AGE (years)	-2.143	3.331	-.050	-.643	.521	-8.707	4.422
	SEX (male vs female)	-97.001	65.522	-.094	-1.480	.140	-226.115	32.113
	BMI (Kg/m^2)	8.038	8.072	.067	.996	.320	-7.868	23.944
	SBP (mmHg)	-.975	2.613	-.032	-.373	.709	-6.125	4.174
	DBP (mmHg)	-5.663	3.749	-.142	-1.511	.132	-13.050	1.724
	GLUCOSE (mg/dl)	-.759	2.267	-.023	-.335	.738	-5.225	3.708
	eGFR MDRD (mL/min/1.73 m^2)	**-5.085**	**1.356**	**-.257**	**-3.750**	**.000**	**-7.758**	**-2.413**
	Nº DRUGS (2 or more vs less that 2)	**185.842**	**81.868**	**.163**	**2.270**	**.024**	**24.515**	**347.169**

Adjusted R Square
.109

If we consider only those patients with UAE<300 mg/day, in this case, the variables that were independently associated with UAE were: fasting glucose (Beta 0.510 (0.44-0.97), sig 0.032) and the number of drugs (Beta 39 (22-56), sig<0.0001). The eGFR by MDRD was no longer independently associated after excluding patients with macroalbuminuria (Table 6.4).

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Table 6. 4. *Result of the linear regression analysis for UAE excluding macroalbuminurics.*

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	-16.114	48.663		-.331	.741	-112.040	79.812
	AGE (years)	-.166	.350	-.036	-.473	.637	-.856	.525
	SEX (male vs female)	-2.007	6.882	-.019	-.292	.771	-15.572	11.559
	BMI	.387	.848	.030	.456	.649	-1.285	2.060
	SBP (mmHg)	.059	.269	.019	.219	.827	-.471	.589
	DBP (mmHg)	-.486	.391	-.114	-1.245	.215	-1.257	.284
	GLUCOSE (mg/dl)	**.510**	**.236**	**.145**	**2.155**	**.032**	**.044**	**.976**
	eGFR MDRD (mL/min/1.73 m^2)	-.183	.146	-.085	-1.255	.211	-.470	.104
	Nº DRUGS (2 or more vs less that 2)	**39.146**	**8.693**	**.319**	**4.503**	**.000**	**22.011**	**56.282**

Adjusted R Square
.150

6.3. Genetic analysis

6.3.1. Quality control parameters

6.3.1.1. Individual Call Rate

The average call rate for the individuals was 94.8%±0.06%. 213 subjects (70.3%) had a call rate higher than 95%, 24(7.9%) between 90 and 95% and 66 (21.8%) had a call rate lower than 90%. These last 66 subjects with an individual call rate lower than 90% were not included in the final analysis. The average call rate after filtering these samples was 98%.

6.3.1.2. SNP Call Rate

Of the 262314 SNPs included in the Affymetrix Nsp chip, 150388 (57.3%) had a call rate higher than 95%, 83555 (31.9%) had a call rate between 90 and 95%, and 28371 (10.8%) had a call rate lower than 90%.12379 (4.7%) markers had less than two alleles and were excluded from the analysis. We decided to use 0.9 (90%) as a threshold for SNP call rate because there were a huge amount of SNPs between 90-95% and because this study was planned as a preliminary analysis to select SNPs which later on we will try to replicate in a larger sample. We are concerned that we are increasing the number of false positive (type I error) but in this way we are reducing the number of false negative results (type II error).

6.3.1.3. Hardy Weinberg Equilibrium

Using a p-value of 0.0001 as a threshold, 6239 SNPs, failed the HWE in the whole sample and therefore they were excluded from the association analysis. These SNPs which were not in HWE were equally distributed along the entire genome with no differences regarding case-control status or gender.

6.3.1.4. Minor allele frequency

Considering a MAF of 0.1, 78369 SNPs were not included in the association analysis. We decided this MAF threshold over other commonly used as 0.05 or 0.01 in order to improve our statistical power due to our small sample size.

6.3.1.5. Missingness

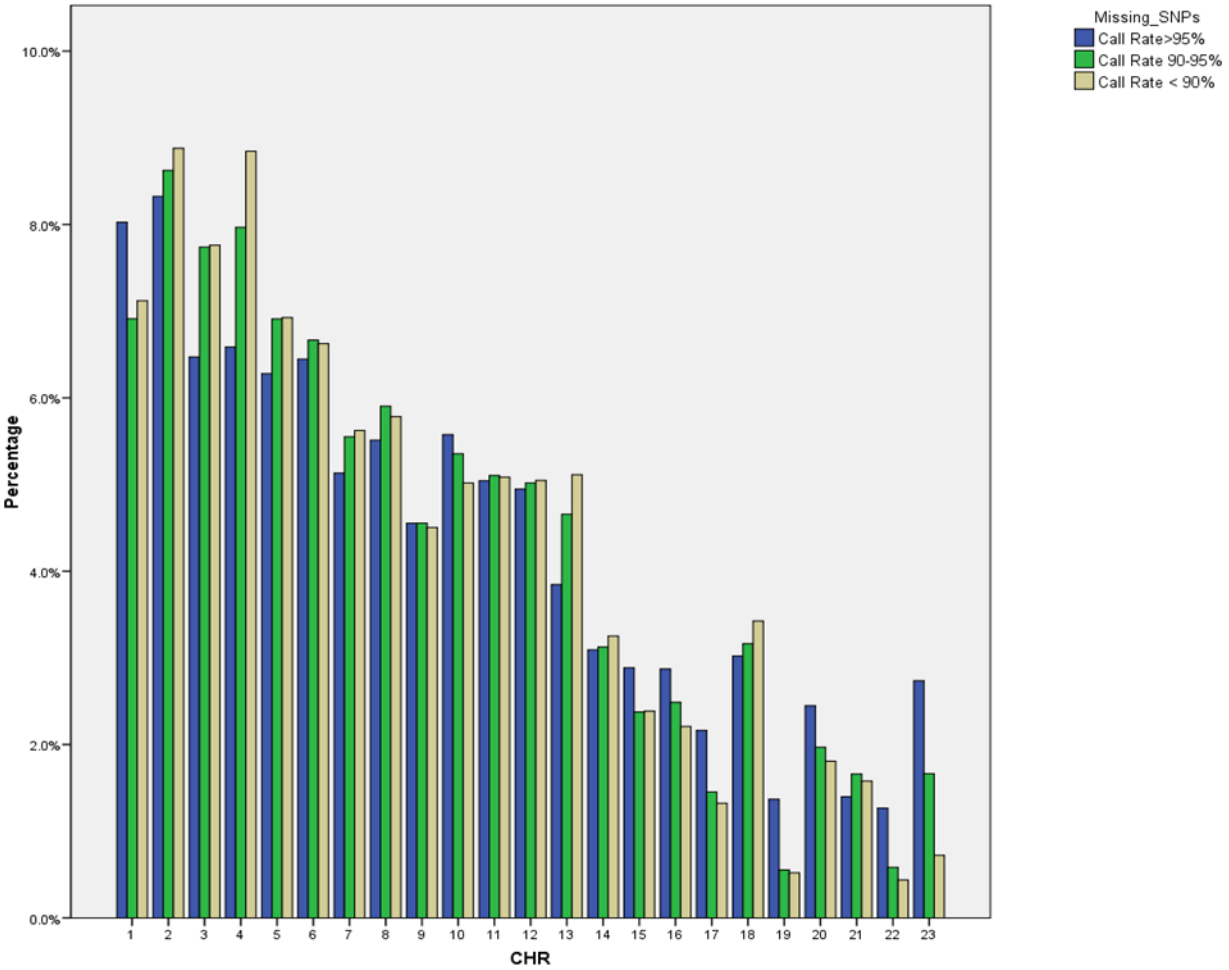
One hundred sixty six SNPs with significant missing rates between cases and controls were not considerer for the association analysis and also 153 SNPs with different missing rates between batches.

6.3.1.6. Total Filtered

After applying the above mentioned filters, 169406 SNPs were selected for the further analysis.

The SNP call rate for each chromosome is shown in figure 6.2.

Figure 6. 2. *Bars diagram of the SNP call rate for each chromosome.*

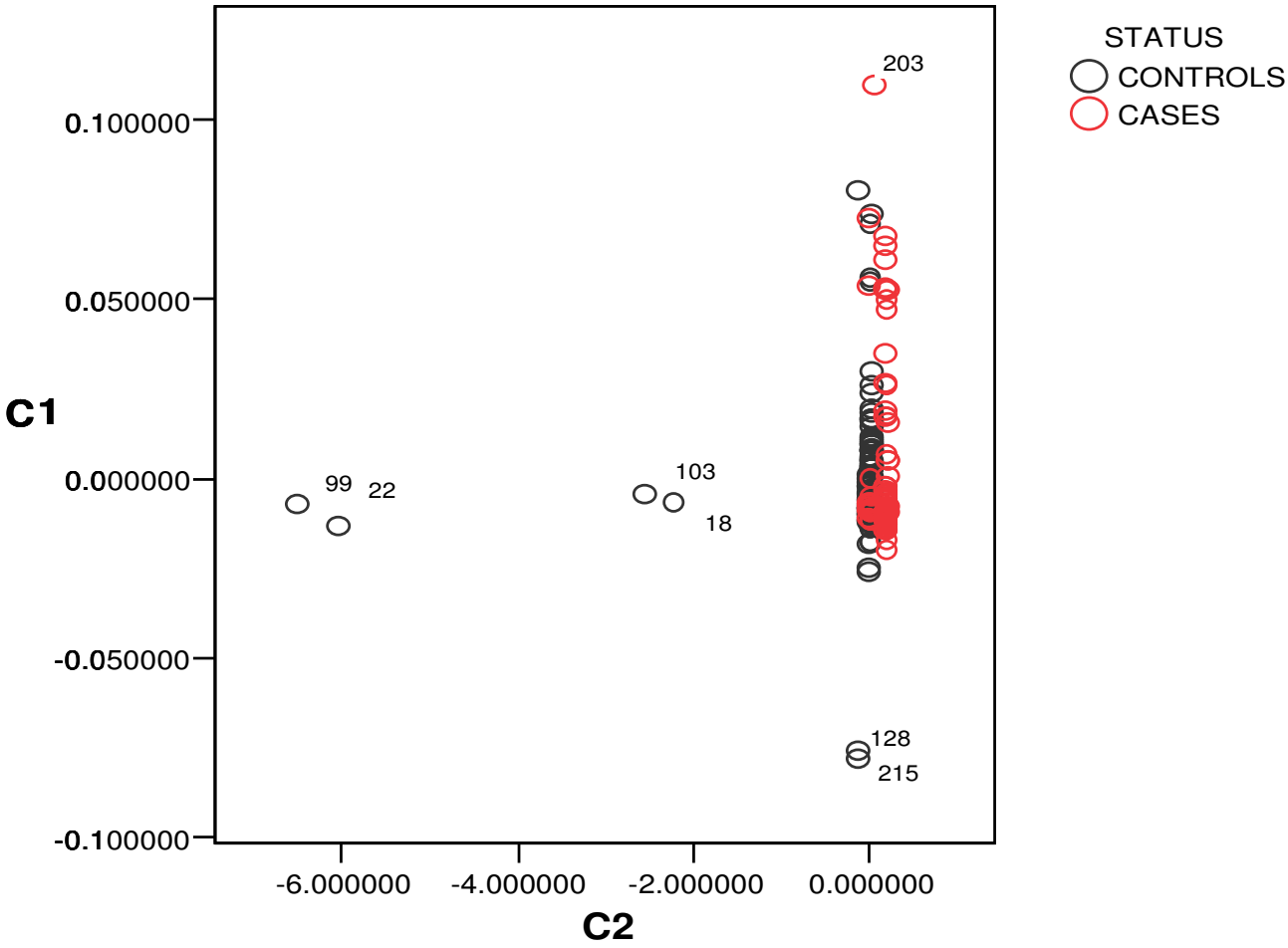


6.3.1.7. Cryptic relatedness

This step performs a check on the cryptic relatedness between study participants. We estimated the sharing of genetic information by estimating identity by state (IBS) using the PLINK software. Surprisingly, we detected 5 individuals with an IBS higher than 95% who were excluded

from the association analysis. We also performed the multidimensional scaling plot for the IBS pairwise distances according to the case-control status in order to detect individuals quite different from the rest and exclude them from the analysis (Figure 6.3)

Figure 6. 3. *Multidimensional scaling plot for the IBS pairwise distances adjusted for the case-control status.*



6.3.1.8. Batch effect

Because we had batches with only cases and batches with only controls we were not able to adjust the analysis taking into account the batch which the individual belongs to. This means that we can not sure that the associated SNPs were due to differences in the genotyping procedure or due to real differences between cases and controls. To solve this problem, we made a database of controls and a database of cases and after that we compared the SNPs between batches in cases and in controls separately. Those SNPs significantly different between batches of cases and those significantly different between batches of controls, were eliminated.

We also deleted those SNPs with different missing rates between batches by comparing the SNP missing rate between each batch and the rest of batches.

6.3.1.9. Sample contamination

As I commented previously, the pairwise clustering based on the Identity by State (IBS) revealed 5 individuals with very high value for the IBS proportion which means that those individuals could be in fact siblings or even twins or correspond to repeat or contaminated samples. One individual of each of these five pairs was excluded for the analysis.

6.3.1.10. Resume

After making all the procedures described above, 77 cases and 152 controls were included in the final analysis. Regarding to the SNPs, 169406 remained after all the filters. The genotyping call rate for these final individuals was 97.9% (range 90.5%-99.6%) and for the SNP was also 97.9% (range 90.4%-100%).

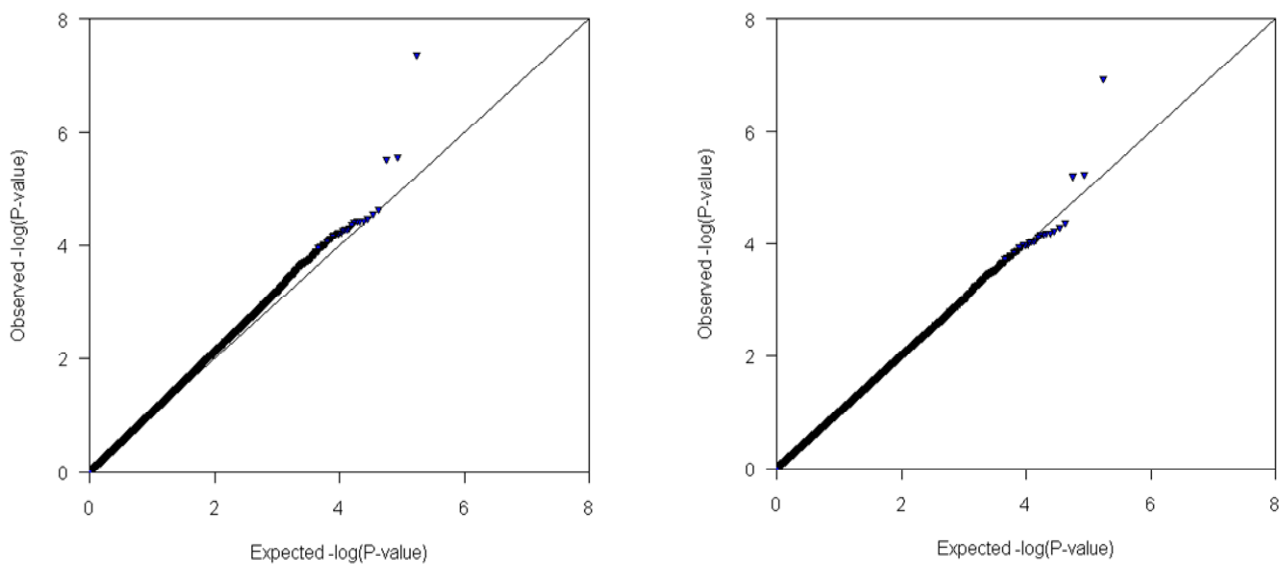
6.3.2. Association analysis for qualitative traits (Unadjusted)

6.3.2.1. Association analysis for albuminuria

The genomic inflation factor (based on the median chi-squared) for the basic association analysis was 1.07. The median chi-square statistic was 1.06.

The Quantile-Quantile (Q-Q) plot for the basis association analysis unadjusted and adjusted for genomic control are shown in the figure 6.4.

Figure 6. 4. *Q-Q Plot for the observed and expected p-values. a) Unadjusted and b) Adjusted for genomic control.*



6.3.2.1.1. Individual SNPs analysis

Between all the selected markers only one SNP [*SNP_A-4215311*(*rs6980941*)] on chromosome 8, cytoband p22, showed significant association even after Bonferroni correction (p-value for the basic allelic test, unadjusted $4.23x10^{-8}$; Bonferroni p-value 0.007; p-value for the trend test 2.21e-007). To assess the consistency of this result, we also performed the adaptative permutation analysis. The *SNP_A-4215311* remained being the most associated marker with an empirical p-value of 1e-006 after 1 million of permutations. The result for this SNP under different genotypic model is shown in the table 6.5.

Table 6. 5. *Association with the main marker on chromosome 8 under different genotypic models.*

Chr	SNP	A1	A2	TEST	AFFECT	UNAFFECT	CHISQ	DF	P-VALUE
8	SNP_A-4215311	A	B	ALLELIC	31/101	15/267	30	1	4.2e-008
8	SNP_A-4215311	A	B	TREND	31/101	15/267	27	1	2.22e-007
8	SNP_A-4215311	A	B	GENO	5/21/40	0/15/126	27	2	1.43e-006
8	SNP_A-4215311	A	B	DOM	26/40	15/126	23	1	1.31e-006
8	SNP_A-4215311	A	B	REC	5/61	0/141	11	1	0.0009379

A1: Minor allele; A2: Major allele.

Carriers of the minor allele of this SNP had significantly higher risk of being albuminuric than carriers of the other allele (OR 5.46 (2.81-10.55), p-value 1.6e-007) according to the Fisher exact test (Table 6.9).

This polymorphism is located in an intergenic region, approximately 100 Kb of distance of the MSR1 (Macrophage acetylated LDL receptor I and II) gene. This gene encodes the class A macrophage scavenger receptors, which include three different types (1, 2, 3) generated by alternative splicing of this gene. These receptors or isoforms are macrophage-specific trimeric integral membrane glycoproteins and have been implicated in many macrophage-associated physiological and pathological processes including atherosclerosis. The isoforms type 1 and type 2 are functional receptors and are able to mediate the endocytosis of modified low density lipoproteins (LDLs).

The other two most associated SNPs were *SNP_A-4204317* (*rs2154401*) on chromosome region 10q11.21 and *SNP_A-1888702* (*rs7974380*) on chromosome region 12q24.23 although they did not reach the statistical significance (p-values for the basic allelic test, 2.69e-006 and 2.89e-006 for *rs2154401* and *rs7974380*, respectively).

Table 6. 6. *Association with the main marker on chromosome 10 under different genotypic models.*

Chr	SNP	A1	A2	TEST	AFFECT	UNAFFECT	CHISQ	DF	P-VALUE
10	SNP_A-4204317	A	B	ALLELIC	95/53	121/177	22	1	2.52e-006
10	SNP_A-4204317	A	B	TREND	95/53	121/177	22	1	2.52e-006
10	SNP_A-4204317	A	B	GENO	32/31/11	20/81/48	26	2	2.24e-006
10	SNP_A-4204317	A	B	DOM	63/11	101/48	8	1	0.005679
10	SNP_A-4204317	A	B	REC	32/42	20/129	25	1	7.09e-007

A1: Minor allele; A2: Major allele.

Table 6. 7. *Association with the main marker on chromosome 12 under different genotypic models.*

Chr	SNP	A1	A2	TEST	AFFECT	UNAFFECT	CHISQ	DF	P-VALUE
12	SNP_A-1888702	A	B	ALLELIC	44/102	149/127	22	1	2.89e-006
12	SNP_A-1888702	A	B	TREND	44/102	149/127	22	1	2.25e-006
12	SNP_A-1888702	A	B	GENO	4/36/33	39/71/28	22	2	1.33e-005
12	SNP_A-1888702	A	B	DOM	40/33	110/28	14	1	0.0001461
12	SNP_A-1888702	A	B	REC	4/69	39/99	15	1	9.31e-005

A1: Minor allele; A2: Major allele.

For the SNP on chromosome 10, cytoband q11.21, the nearest gene, about 250 kb of distance, correspond to the CXCL12 gene. This gene is called "chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)" and belongs to the intercrine family. It has a role in the activation of leukocytes and it induces by different pro-inflammatory stimuli such as lipopolysaccharide, TNF or IL1.

The SNP on chromosome region 12q24.23 is close to several genes (about 150-200Kb) none of them being a suitable candidate gene for albuminuria.

A graphical representation of the main association analysis is shown in the figure 6.5

Figure 6. 5. *Manhattan plot for the main association analysis with albuminuria and cluster plots of the main associated markers.*

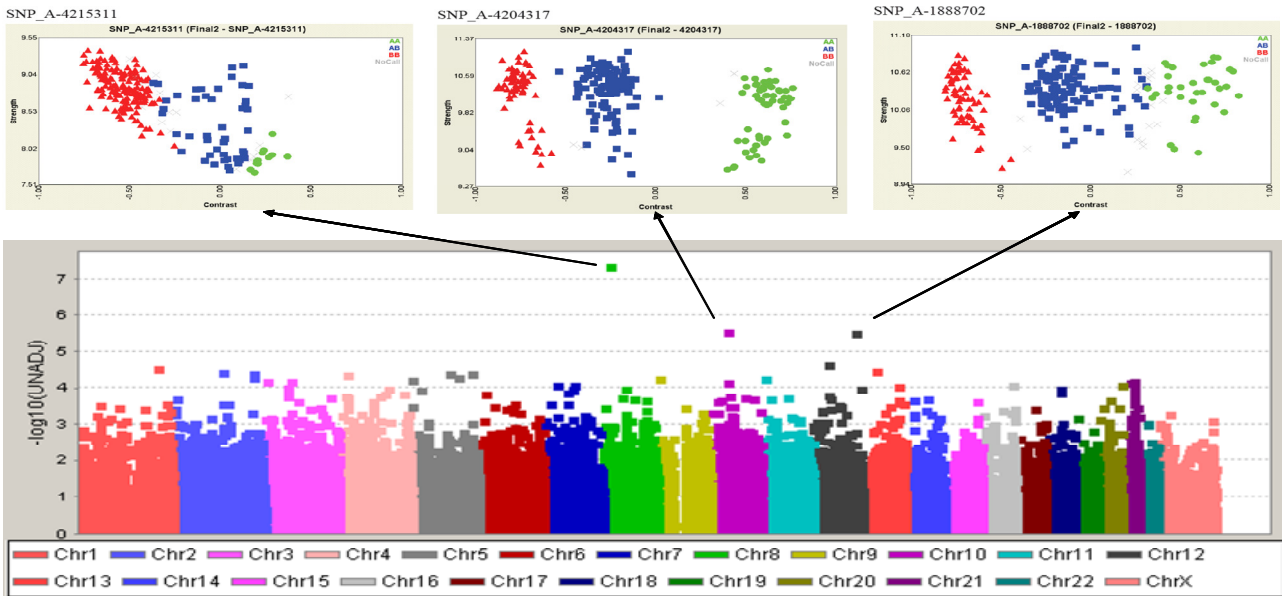


Table 6. 8. *Characteristics of the main associated SNPs with albuminuria.*

SNPID	Call Rate	%AA	%AB	%BB	MAF	H.W. p-Value	dbSNP rS ID	Chr	Physical Position	Allele A	Allele B
SNP_A-4215311	91.67	5.263	19.736	66.666	0.1650	0.00155	rs6980941	8	16185825	C	G
SNP_A-4204317	98.25	24.56	46.49	27.19	0.4866	0.42858	rs2154401	10	43975281	A	G
SNP_A-1888702	92.54	15.35	47.80	29.38	0.4241	0.40359	rs7974380	12	116877645	A	G

Table 6. 9. *Fisher test for the main associated SNPs.*

Chr	SNP	BP	A1	FA	FU	A2	OR	UL	LL	p-val
8p22	SNP_A-4215311	16185825	A	0.235	0.053	B	5.46	2.81	10.55	1.646e-007
10q11.21	SNP_A-4204317	43975281	A	0.642	0.406	B	2.62	1.74	3.94	3.297e-006
12q24.23	SNP_A-1888702	116877645	A	0.301	0.54	B	0.36	0.24	0.56	3.356e-006

A1: Minor allele; A2: Major allele; FA: Frequency in affected; FU: Frequency in unaffected; UL: Upper limit, LL: Lowe limit.

6.3.2.1.2. Haplotype and LD analysis

In this region 8p22 we found one haplotype, which included the marker most associated with the risk of albuminuria. This haplotype, *BAA*, include the allele *A* of the previously associated SNP, the allele *B* for the *SNP_A-1802309*, which is located -142 kb to the main associated marker, and the allele *A* for the *SNP_A-2202211*, which is located -101 kb to the main associated SNP. These two SNPs are located within intronic regions of the MSR1 gene.

It is noteworthy that the association with the haplotype is stronger than the association with the individual SNP (Table 6.10).

Table 6. 10. *Haplotype association analysis for markers in region 8p22.*

SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	OR	CHISQ	P-VALUE
SNP_A-1802309 (rs351562)	0.237/0.233	0.996	-142	0.0195	1.19	0.591	0.442
SNP_A-2202211 (rs614439)	0.165/0.125	0.991	-101	0.0509	1.23	0.668	0.414
SNP_A-4215311 (rs2154401)	**0.111/0.042**	**0.904**	**0**	*	**4.7**	**26.6**	**2.47e-007**

HAPLOTYPE ..*	FREQ	OR	CHISQ	P-VALUE
BAA	**0.0655**	**6.82**	**28**	**1.2e-007**
ABB	0.0732	1.16	0.22	0.639
AAB	0.119	0.839	0.375	0.54
BAB	0.647	0.548	6.74	0.00944

SNP_A-1802309, SNP_A-2202211, **SNP_A-4215311**

Figure 6. 6. *Cluster plots for the other two markers which form the haplotype with the most associated marker.*

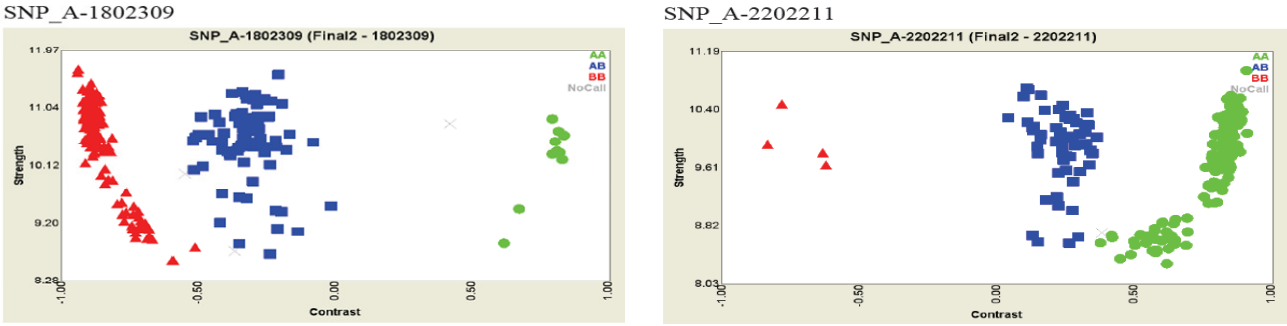
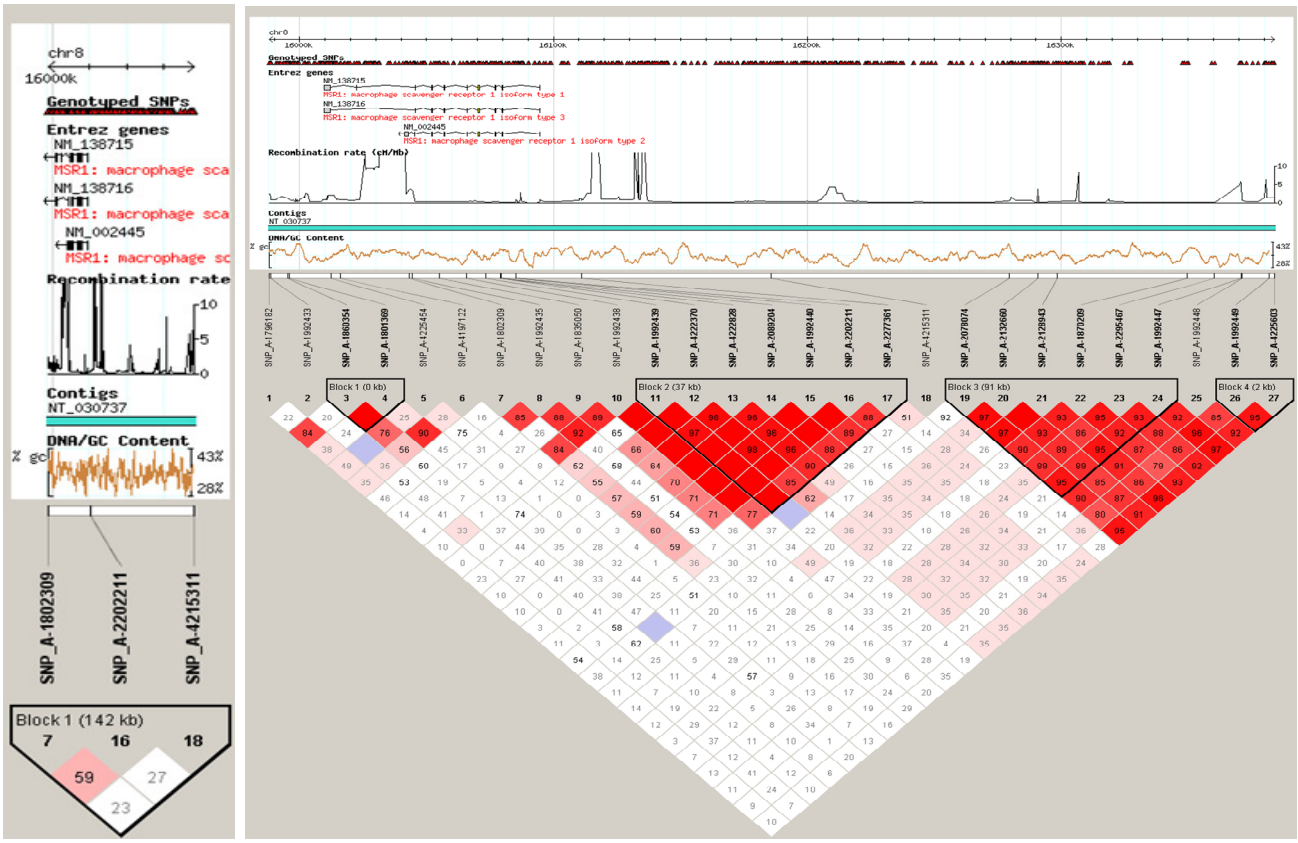


Table 6. 11. *Main characteristics of the other two markers which form the haplotype with the most associated marker.*

SNPID	SNP Call Rate	SNP %AA	SNP %AB	SNP %BB	MAF	H.W. p-Value	dbSNP rs ID	Chr	Physical Position	Allele A	Allele B
SNP_A-1802309	98.68	4.38	35.52	58.77	0.224	0.609	rs351562	8	16043410	A	G
SNP_A-2202211	99.56	72.36	25.43	1.75	0.145	0.670	rs614439	8	16085267	C	G

The LD of the associated region on chromosome 8 is shown in figure 6.7.

Figure 6. 7. *LD map with haploblocks for the region ± 200kb of the hit on chromosome 8 (LD is shown as D'/LOD with the D' values).*



None of the proxy SNPs to the most associated one (*SNP_A-4215311*) were in strong linkage (R^2>0.8) with it.

As we can see in the figure above, the main associated marker (*SNP_A-4215311* (*rs6980941*) is located between two areas of high LD. In contrast the *SNP_A-2202211* (rs614439) is a region of high LD and is a Tag-SNPs for the following markers: *SNP_A-1992440* (rs614794), *SNP_A-4222370* (*rs438721*), *SNP_A-4222828* (*rs368773*), *SNP_A-2089204* (*rs414580*).

For the marker on chromosome region 10q11.21, the haplotype association was weaker than that for the individual marker (Table 6.12)

Table 6. 12. *Haplotype association analysis for markers in region 10q11.21.*

SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	OR	CHISQ	P-VALUE
SNP_A-2213753 (rs1492712)	0.293/0.258	0.969	-123	0.245	0.563	7.52	0.00611
SNP_A-2001107 (rs2639463)	0.468/0.450	0.956	-89.2	0.64	2.23	15.5	8.1e-005
SNP_A-4204317 (rs10810668)	**0.484/0.408**	**0.974**	**0**	*****	**2.6**	**22.7**	**1.93e-006**

HAPLOTYPE ..*	FREQ	OR	CHISQ	P-VALUE
BBA	**0.406**	**2.36**	**17.6**	**2.77e-005**
BAA	0.0501	2.01	3.61	0.0573
AAB	0.264	0.505	9.71	0.00183
BAB	0.209	0.474	9.09	0.00258

SNP_A-2213753, SNP_A-2001107, **SNP_A-4204317**

All the SNP forming the haplotype are in an intergenic region of chromosome 10, cytoband q11.21. The LD of that region is shown in the figure 6.8.

Figure 6. 8. *LD of the associated region on chromosome 10.*

The main associated marker in this region is not in high LD (R-sq>0.8) with others markers.

For the markers on chromosome 12, cytoband q24.23, the haplotype analysis and LD is shown in table 6.13 and figure 6.9.

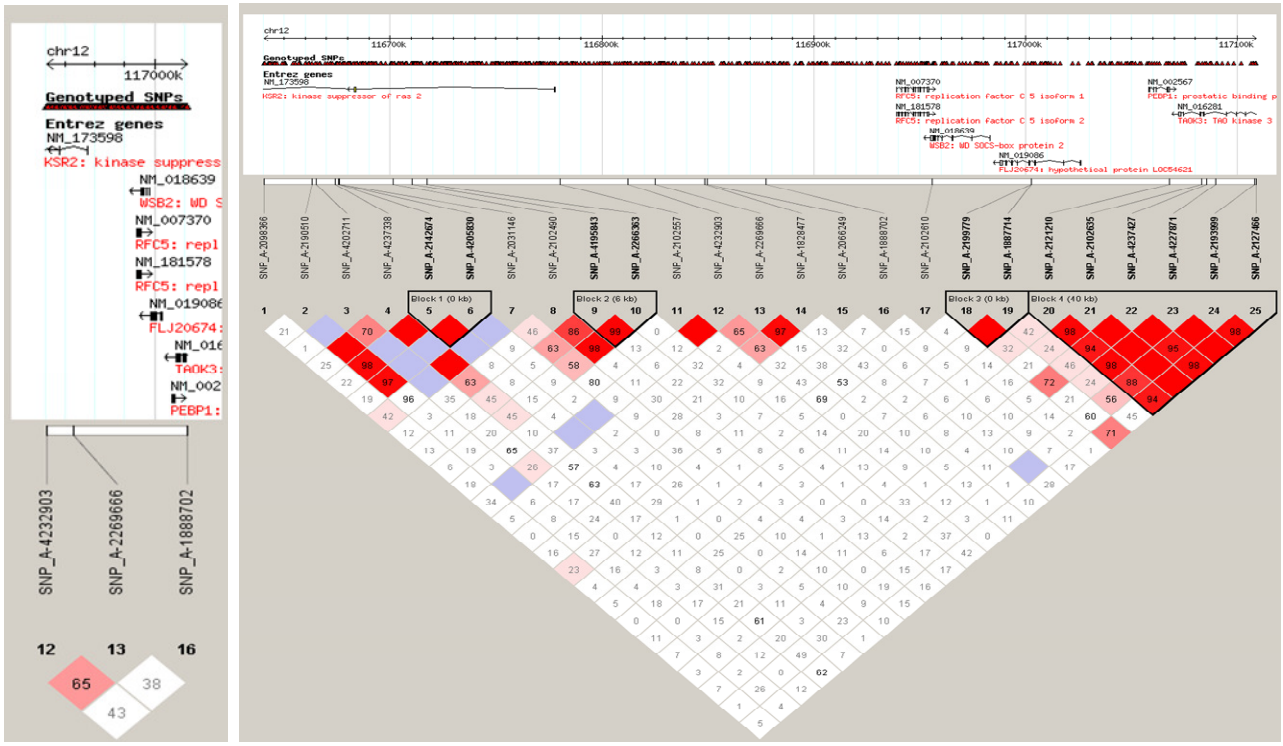
Table 6. 13. *Haplotype association analysis for markers in region 12q24.23.*

SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	OR	CHISQ	P-VALUE
SNP_A-4232903 (rs11068715)	0.114/0.075	1	-65.3	0.0204	1.16	0.233	0.629
SNP_A-2269666 (rs10850931)	0.122/0.067	1	-52.5	0.0184	1.44	1.68	0.195
SNP_A-1888702 (rs7974380)	**0.457/0.425**	**0.921**	**0**	*	**0.394**	**22.6**	**2e-006**

HAPLOTYPE ..*	FREQ	OR	CHISQ	P-VALUE
ABA	**0.413**	**0.421**	**20.5**	**6.08e-006**
BAB	0.06	1.91	3.43	0.0639
ABB	0.43	1.95	12.7	0.000365
BAB	0.209	0.474	9.09	0.00258

SNP_A-4232903, SNP_A-2269666, **SNP_A-1888702**

Figure 6. 9. *LD of the associated region on chromosome 12 cytoband q24.23.*

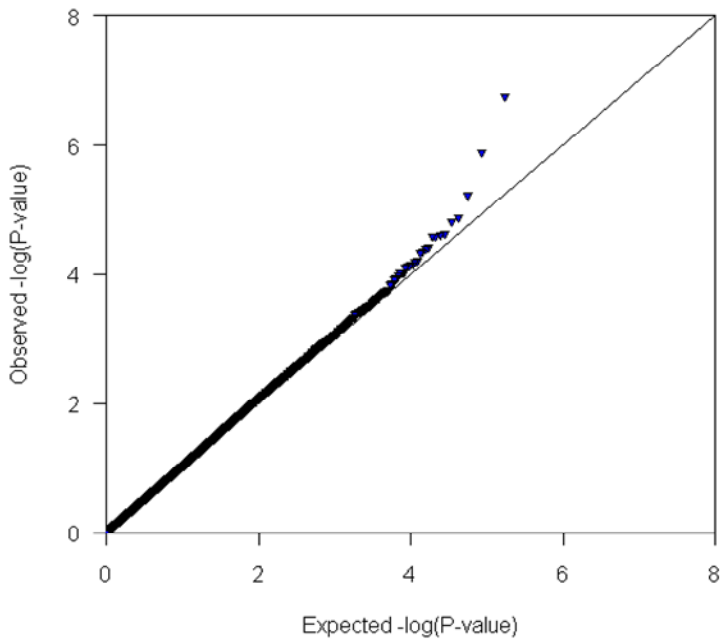


Again, there are not other SNPs in high LD with the associated marker in this region.

6.3.2.2. Association analysis for microalbuminuria

The genomic control for this analysis was 1.05 with a mean chi-square statistic of 1.04.

Figure 6. 10. *Q-Q plot for the observed and expected p-values (unadjusted) for the association with microalbuminuria.*



6.3.2.2.1. Individual SNPs analysis

After removing 8 patients with macroalbuminuria (EUA≥300mg/day), the most associated SNP on chromosome 8 in the individual analysis without remained statistically significant even considering Bonferroni correction [*SNP_A-4215311*(*rs6980941*), unadjusted p-value 1.74e-007; Bonferroni p-value 0.029 for the basic allelic test]. The second and third most associated SNP were also the same that in the association analysis for albuminuria, *SNP_A-4204317* (*rs2154401*) on chromosome region 10q11.21 and *SNP_A-1888702* (*rs7974380*) on chromosome region 12q24.23. As it happened before these SNP did not reach the statistical significance (p-value unadjusted 1.28e-006 and 6.12e-006, Bonferroni p-value 0.21 and 1, respectively).
The association with these markers under different genotypic model is shown in tables 6.14-6.16. The Manhattan plot for this analysis is shown in figure 6.11.

Table 6. 14. *Association with the main marker on chromosome 8 under different genotypic models.*

Chr	SNP	A1	A2	TEST	AFFECT	UNAFFECT	CHISQ	DF	P-VALUE
8	SNP_A-4215311	A	B	ALLELIC	27/91	15/267	27	1	1.739e-007
8	SNP_A-4215311	A	B	TREND	27/91	15/267	25	1	5.966e-007
8	SNP_A-4215311	A	B	GENO	4/19/36	0/15/126	25	2	3.61e-006
8	SNP_A-4215311	A	B	DOM	23/36	15/126	22	1	3.16e-006
8	SNP_A-4215311	A	B	REC	4/55	0/141	10	1	0.001789

A1: Minor allele; A2: Major allele.

Table 6. 15. *Association with the main marker on chromosome 10 under different genotypic models.*

Chr	SNP	A1	A2	TEST	AFFECT	UNAFFECT	CHISQ	DF	P-VALUE
10	SNP_A-4204317	A	B	ALLELIC	87/45	121/177	23	1	1.27e-006
10	SNP_A-4204317	A	B	TREND	87/45	121/177	24	1	9.429e-007
10	SNP_A-4204317	A	B	GENO	29/29/8	20/81/48	27	2	1.55e-006
10	SNP_A-4204317	A	B	DOM	58/8	101/48	10	1	0.001959
10	SNP_A-4204317	A	B	REC	29/37	20/129	24	1	8.653e-007

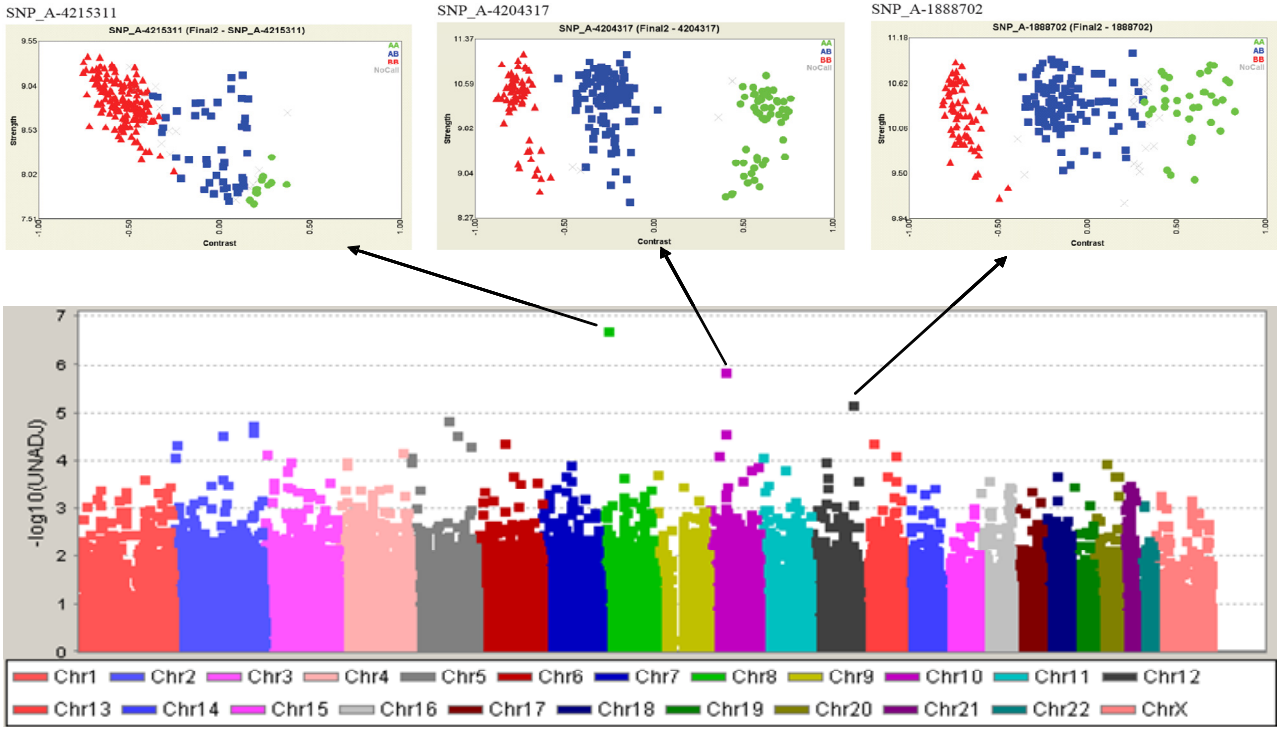
A1: Minor allele; A2: Major allele.

Table 6. 16. *Association with the main marker on chromosome 12 under different genotypic models.*

Chr	SNP	A1	A2	TEST	AFFECT	UNAFFECT	CHISQ	DF	P-VALUE
12	SNP_A-1888702	A	B	ALLELIC	39/91	149/127	20	1	6.12e-006
12	SNP_A-1888702	A	B	TREND	39/91	149/127	21	1	5.47e-006
12	SNP_A-1888702	A	B	GENO	4/31/30	39/71/28	21	2	3.3e-005
12	SNP_A-1888702	A	B	DOM	35/30	110/28	14	1	1.41e-004
12	SNP_A-1888702	A	B	REC	4/61	39/99	13	1	3.23e-004

A1: Minor allele; A2: Major allele.

Figure 6. 11. *Manhattan plot for the association analysis with microalbuminuria.*



6.3.2.2.2. Haplotype and LD analysis

The association with the three SNPs haplotype "*BAA*" on chromosome region 8p22 remained but it was a little bit weaker than the previously described for albuminuria as you can see in table 6.17.

Table 6. 17. *Haplotype association analysis for the markers on chromosome region 8p22.*

SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	OR	CHISQ	P-VALUE
SNP_A-1802309 (rs351562)	0.239/0.233	0.995	-142	0.0212	1.24	0.889	0.346
SNP_A-2202211 (rs614439)	0.168/0.125	0.995	-101	0.0526	1.29	0.956	0.328
SNP_A-4215311 (rs6980941)	**0.105/0.042**	**0.905**	**0**	*****	**4.54**	**24.4**	**7.97e-007**

HAPLOTYPE ..*	FREQ	OR	CHISQ	P-VALUE
BAA	**0.0597**	**6.76**	**24.5**	**7.51e-007**
ABB	0.0741	1.27	0.49	0.484
AAB	0.121	0.899	0.128	0.721
BAB	0.649	0.604	5.34	0.0209

SNP_A-1802309, SNP_A-2202211, **SNP_A-4215311**

Regarding to the haplotype on chromosome region 10q11.21, the association with the haplotype, *BBA*, although it was weaker than that for the individual SNP, it was stronger than that for albuminuria (Table 6.18).

Table 6. 18. *Haplotype association analysis for the markers on chromosome region 10q11.21.*

SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	OR	CHISQ	P-VALUE
SNP_A-2213753 (rs1492712)	0.297/0.258	0.968	-123	0.244	0.576	6.65	0.00989
SNP_A-2001107 (rs2639463)	0.469/0.450	0.955	-89.2	0.653	2.41	17.1	2.61e-005
SNP_A-4204317 (rs10810668)	**0.484/0.408**	**0.973**	**0**	*	**2.8**	**24.6**	**7.13e-007**

HAPLOTYPE ..*	FREQ	OR	CHISQ	P-VALUE
BBA	**0.407**	**2.94**	**23.7**	**1.13e-006**
AAB	0.267	0.52	8.48	0.00359
BAB	0.208	0.435	10.3	0.00136

SNP_A-2213753, SNP_A-2001107, **SNP_A-4204317**

In contrast the association with the haplotype, *ABA*, on chromosome 12, cytoband q24.23, was weaker than that obtained for albuminuria (Table 6.19).

Table 6. 19. *Haplotype association analysis for the markers on chromosome region 12q24.23.*

SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	OR	CHISQ	P-VALUE
SNP_A-4232903 (rs11068715)	0.115/0.075	1	-65.3	0.0229	1.23	0.465	0.495
SNP_A-2269666 (rs10850931)	0.124/0.067	1	-52.5	0.0206	1.56	2.4	0.122
SNP_A-1888702 (rs7974380)	**0.463/0.425**	**0.919**	**0**	*	**0.393**	**20.9**	**4.93e-006**

HAPLOTYPE ..*	FREQ	OR	CHISQ	P-VALUE
ABA	**0.419**	**0.425**	**18.3**	**1.87e-005**
BAB	0.0607	2.05	4.05	0.0441
ABB	0.421	1.88	10.7	0.00107

SNP_A-4232903, SNP_A-2269666, **SNP_A-1888702**

6.3.3. Association analysis for qualitative traits adjusted for clinical co-variables

Before to start the description of these analyses, it is important to say that there were a significant number of subjects with missing values for some of the co-variables (around 54 depending on the model), so the sample size and therefore the statistical power were markedly reduced.

6.3.3.1. Association analysis for albuminuria

This analysis included only 38 cases and 137 controls. The genomic controls for these analysis was around 1.0 and the mean chi-square statistic was also around 1.0.

In this case, there was not association for the marker, *SNP_A-4215311* (p-value 0.06), which was significantly associated with albuminuria without including clinical co-variables neither for the haplotype "*BAA*" of the SNPs: *SNP_A-1802309*, *SNP_A-2202211*, *SNP_A-4215311* (p-value 0.07).

As co-variables we included those clinical variables that seem to increase the risk of albuminuria including: age, sex, BMI, SBP, glucose, creatinine clearance (CC) estimated by Cockcroft-Gault formula, eGFR by MDRD formula and the number of drugs expressed as a qualitative variable (two or more drugs or less than two drugs).

6.3.3.1.1. Models with number of drugs as co-variable

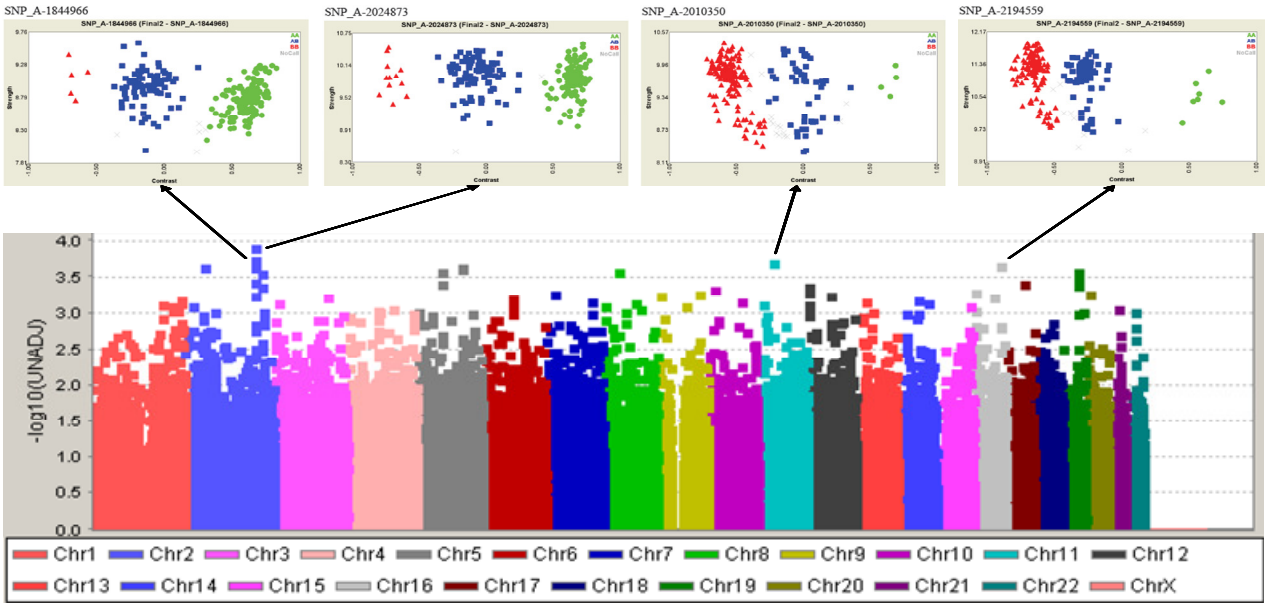
6.3.3.1.1.1. Individual SNPs analysis

For the models which include the NºDrugs plus all the others important co-variables, the first and second most associated SNPs were on chromosome 2, cytoband q32.2, but they were very far from significance, *SNP_A-1844966* (*rs6719224*), and *SNP_A-2024873* (*rs2033870*), unadjusted p-value 0.00018 and 0.00019 respectively. One of them is located between the O-sialoglycoprotein endopeptidase-like 1 (OSGLPL1) and the ORM1-like1 (ORMDL1) genes and the other within an intron of the Ankyrin and armadillo repeat-containing protein (ANKAR) gene.

The third most associated SNP in this analysis was on chromosome 11, cytoband p12 [*SNP_A-2010350* (*rs3552231*), unadjusted p-value=0.00019] in an intergenic region without any known gene close to it. The fourth most associated marker [*SNP_A-2194559* (*rs146509*), unadjusted p-value= 0.00021] was on chromosome 16, cytoband q23.1, in an intergenic region of the WW domain containing oxidoreductase (WWOX) gene.

The Manhattan plot for these results is shown in Figure 6.12.

Figure 6. 12. *Manhattan plot for the association analysis with albuminuria including age, sex, BMI, SBP, glucose, eGFR (MDRD) and number of drugs as co-variables.*



6.3.3.1.1.2. Haplotype and LD analysis

We did not find association with any haplotype including the two associated markers on chromosome 2, cytoband q32.2. The most associated haplotype in that region of chromosome 2 is shown in table 6.20 and include alleles of two SNP within intronic regions of the ANKAR gene and of another polymorphism close to asparagine synthetase domain-containing protein 1 (ASNSD1) and the solute carrier family 40 (iron-regulated transporter), member 1 (SLC40A1) genes.

Table 6. 20. *Haplotype association analysis for the markers on chromosome region 2q32.2.*

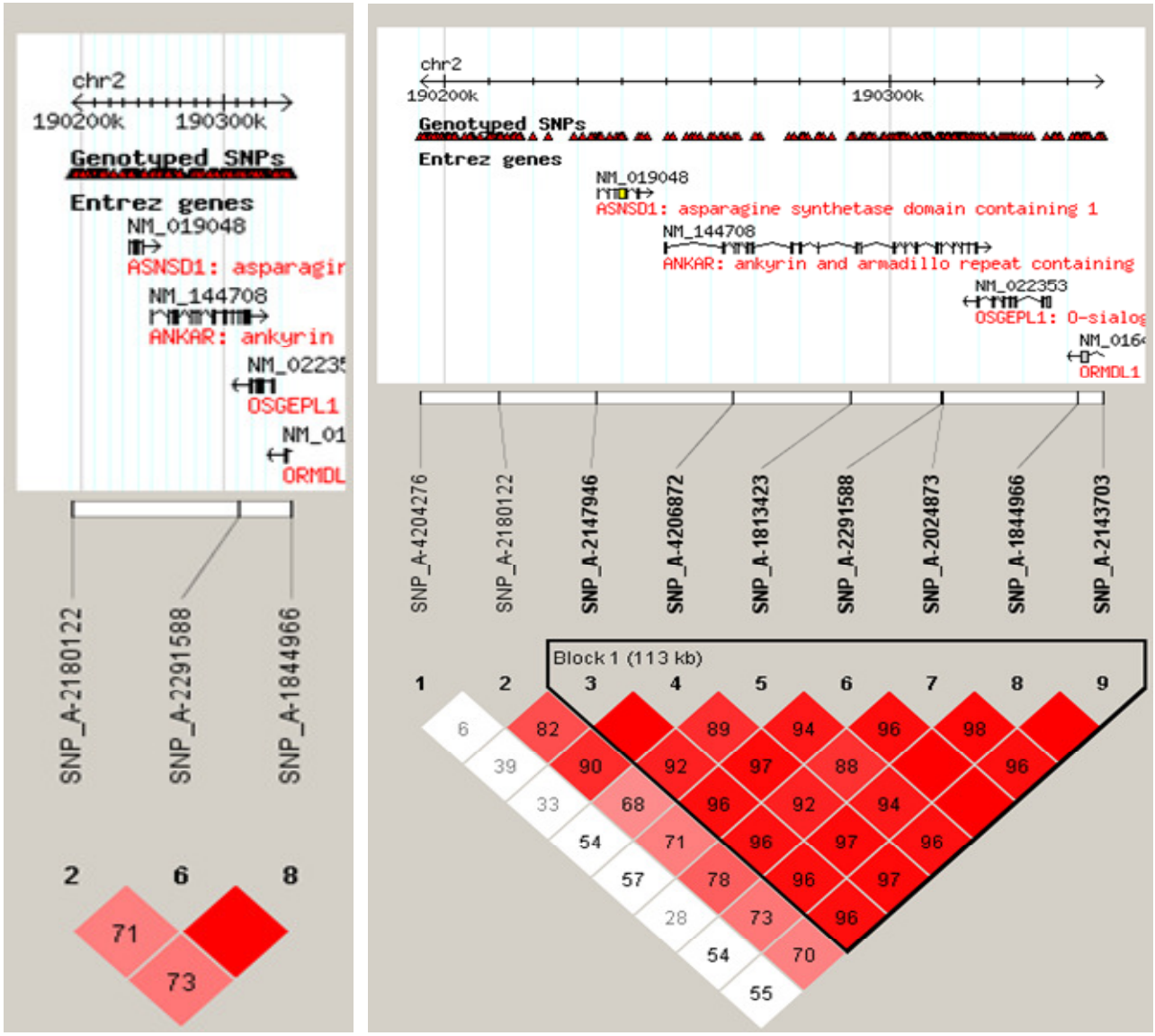
SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	OR	CHISQ	P-VALUE
SNP_A-2180122 (rs4667295)	0.232/0.200	0.987	-130	0.421	0.452	5.77	0.0163
SNP_A-2291588 (rs1550388)	0.199/0.146	1	-30.5	1	0.161	16.4	5.05e-005
SNP_A-1844966 (rs6719224)	**0.195/0.225**	**0.965**	**0**	*****	**0.161**	**16.4**	**5.05e-005**

HAPLOTYPE ..*	FREQ	OR	CHISQ	P-VALUE
BAB	**0.155**	**0.148**	**14.7**	**1.27e-004**
BBA	0.0766	1.52	1.04	0.307
ABA	0.725	2.34	6.25	0.0124

SNP_A-2180122, SNP_A-2291588, **SNP_A-1844966**

The LD of that region on chromosome 2 is shown in figure 6.13.

Figure 6. 13. *LD of the associated region on chromosome 2.*



As we expected the two most associated markers (*SNP_A-2024873* and *SNP_A-1844966*, first and second position) on chromosome region 2q32.2 are in high LD (R-sq=1).

There is a Tag SNP (*SNP_A-2143703*) within an intronic region of the ORM1-like 1 (ORMDL1) gene which captures two of the SNPs of the above described haplotype.

The strength of association for the proxy haplotype analysis on chromosome region 11p12 was very weak (Table 6.21).

Table 6. 21. *Haplotype association analysis for the markers on chromosome region 11p22.*

SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	OR	CHISQ	P-VALUE
SNP_A-1784634 (rs7929217)	0.149/0.117	0.965	-103	0.302	2.05	4.25	0.0393
SNP_A-2010350 (rs355231)	**0.129/0.083**	**0.965**	**0**	*****	**2.63**	**7.78**	**0.00529**
SNP_A-2296741 (rs996262)	0.281/0.316	0.978	79.1	0.337	1.88	5.56	0.0184

HAPLOTYPE .*.	FREQ	OR	CHISQ	P-VALUE
BAB	**0.0799**	**2.61**	**6.11**	**0.0134**
ABB	0.145	1.36	0.832	0.362
ABA	0.66	0.519	4.18	0.0409

SNP_A-1784634, **SNP_A-2010350**, SNP_A-2296471

The LD of this region on chromosome 11 is shown in figure 6.14.

Figure 6. 14. *LD of the associated region on chromosome 11.*

The main associated marker in this region is inside one haploblock but there are not others SNPs in high LD with it (R-sq>0.8). As it can see in the figure above there are not genes in that region.

For the marker in region q23.1 of chromosome 16, we did not find any haplotype associated (Table 6.22).

Table 6. 22. *Haplotype association analysis for the markers on chromosome region 16q23.1.*

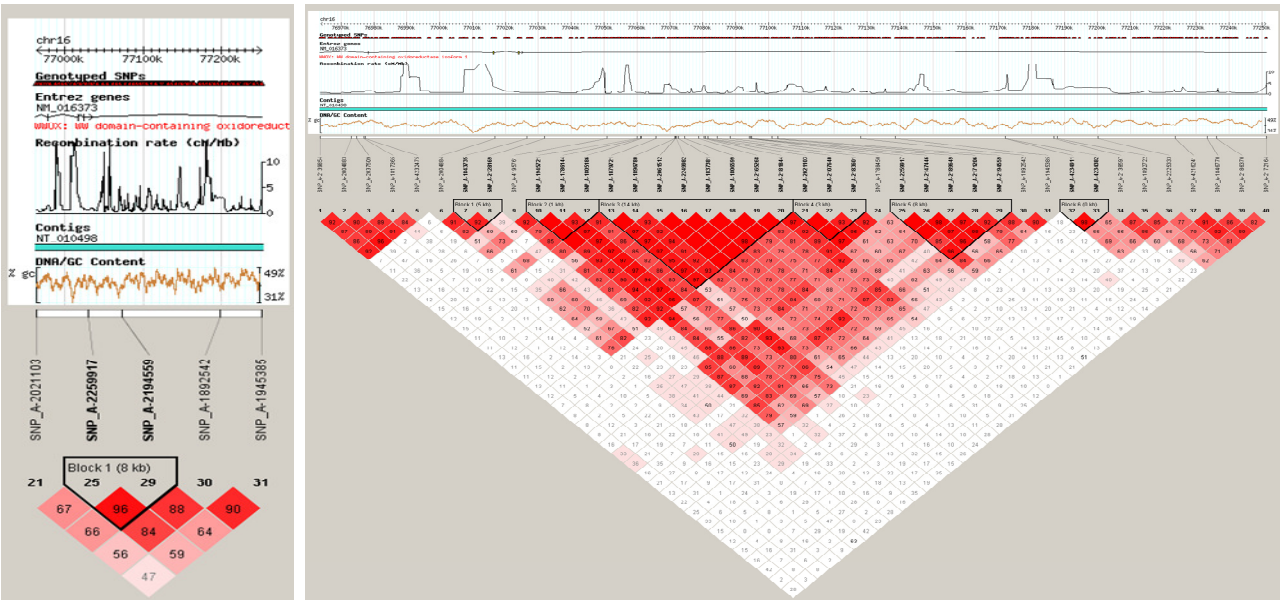
SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	OR	CHISQ	P-VALUE
SNP_A-2021103 (rs2738708)	0.132/0.188	0.996	-22	0.283	1.42	0.899	0.343
SNP_A-2259917 (rs11150082)	0.171/0.172	0.983	-8.65	0.815	1.53	1.74	0.187
SNP_A-2194559 (rs1465099)	0.192/0.186	0.978	0	*	1.93	5.05	0.0246
SNP_A-1892542 (rs2738502)	0.312/0.312	0.987	25.2	0.44	1.39	1.4	0.237
SNP_A-1945385 (rs2667648)	0.201/0.200	1	35.8	0.425	1.23	0.428	0.513

HAPLOTYPE ..*..	FREQ	OR	CHISQ	P-VALUE
BBABA	0.0728	1.49	0.816	0.366
AABBB	0.0741	1.24	0.176	0.675
ABBAB	0.635	0.703	0.579	0.447

SNP_A-2021103, SNP_A-2259917, SNP_A-2194559, SNP_A-1892542, SNP_A-1945385

The LD of that region of the WWOX gene on chromosome 16 is shown in figure 6.15.

Figure 6. 15. *LD of the associated region on chromosome 16.*



The most associated marker in that region, *SNP_A-2194559* is a tag-SNP for *SNP_A2259917* (R-sq=0.808). Other marker within the haplotype (*SNP_A-2021103*) is also a tag-SNP for other five polymorphisms.

6.3.3.1.2. Models without number of drugs as co-variable

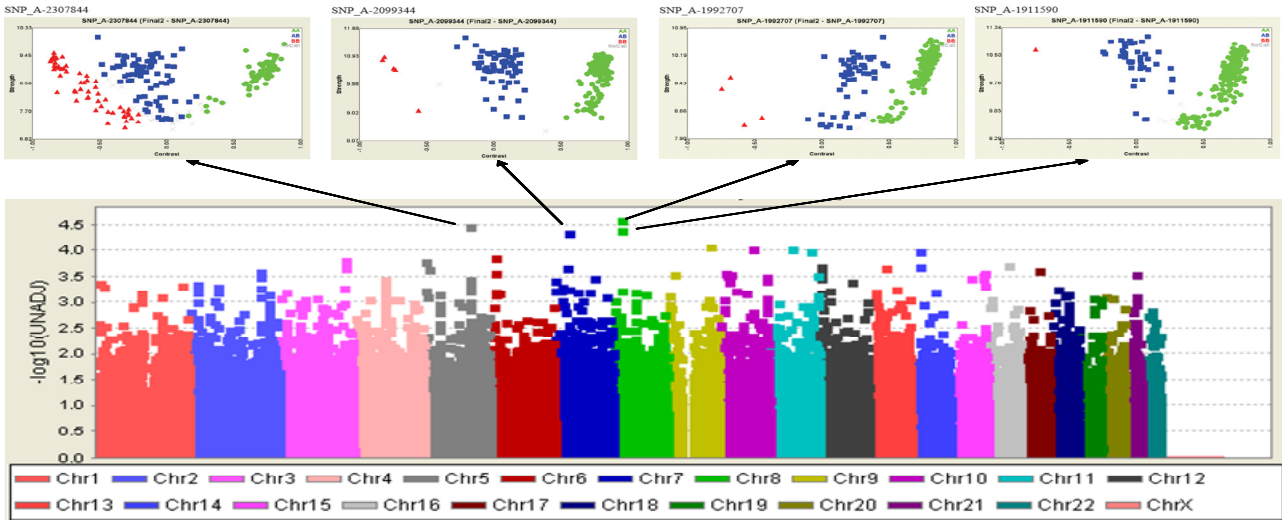
6.3.3.1.2.1. Individual SNPs analysis

For the model without NºDrugs as co-variable, the results were different. These results remained quite similar it does not matter that we use the CC estimated by the Crockoft-Gault formula, the eGFR by MDRD or the DBP instead SBP. The most associated SNP in this case, although without statistical significance after multiple comparisons correction was also located in the short arm of chromosome 8 (cytoband p21.2) and correspond to the marker *SNP_A-1992707* (*rs4872244*) (unadjusted p-value 3.219e-005). The third most associated SNP [*SNP_A-1911590* (*rs6557750*)] was also in the same location 8p21.2. These two SNPs are in LD with an R-sq value=0.641 and D'= 0.836. These two SNPs on chromosome region 8p21.2 are quite close (less than 100kb) to the ADAM7 gene. This gene belongs to a family of zinc-binding proteins which can act either as adhesion proteins or as endopeptidases. Therefore can be involved in multiple biological processes including inflammation.

The second most associated marker, was *SNP_A-2307844* (*rs11241841*), unadjusted p-value 3.33e-005 which was located on chromosome 5, cytoband q23.2, in an intergenic region without genes close to it. The fourth most associated marker was *SNP_A-2099344* (*rs1721936*) unadjusted p-value 4.31e-005, on chromosome 7, cytoband p14.1, close to several genes: EPDR1, SFRP4, TXNDC3.

The Manhattan plot for these results is shown in figure 6.16.

Figure 6. 16. *Manhattan plot for the association analysis with albuminuria including age, sex, BMI, SBP, glucose, and eGFR (MDRD) as co-variables.*



6.3.3.1.2.2. Haplotypes and LD analysis

For the marker on chromosome region 8p21.2, we also found one haplotype including the two previously described markers and the marker [*SNP_A-4225817* (*rs12551522*)] which is located within an intronic region of the ADAM7 gene. This SNP is also a Tag-SNP for the marker *SNP_A-2090729* (*rs2131227*). The association with the haplotype is a little bit stronger than the individual SNPs association (Table 6.23).

Table 6. 23. *Haplotype association analysis for the markers on chromosome region 8p21.2.*

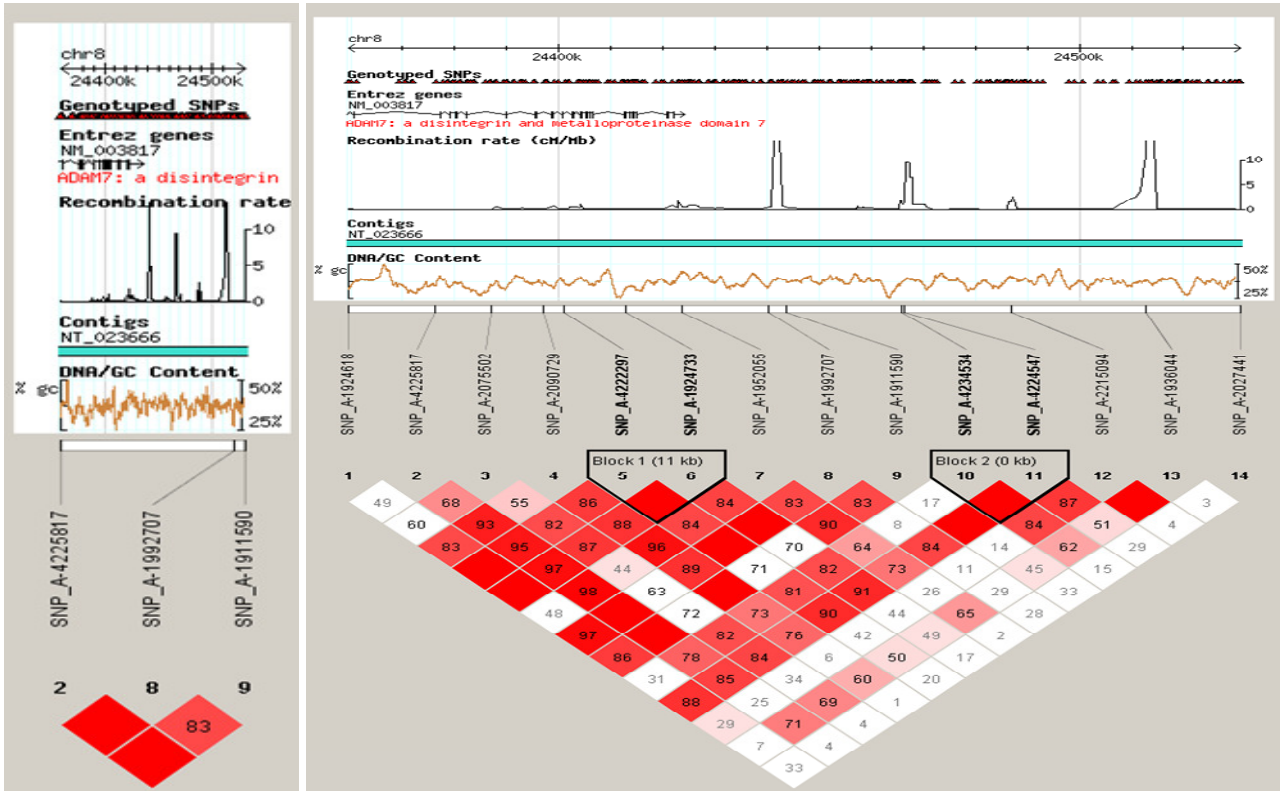
SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	OR	CHISQ	P-VALUE
SNP_A-4225817 (rs12551522)	**0.382/0.30**	**0.978**	**-67.4**	**0.0769**	**1.18**	**0.662**	**0.416**
SNP_A-1992707 (rs4872244)	0.124/0.229	0.969	-3.47	0.639	2.26	8.01	0.00466
SNP_A-1911590 (rs6557750)	0.11/0.225	0.991	0	*	2.25	7.95	0.00481

HAPLOTYPE .*.	FREQ	OR	CHISQ	P-VALUE
ABB	0.0952	1.91	4.43	0.0353
AAB	0.0159	5.16	4.83	0.028
ABA	0.0254	3.46	4.8	0.0284
BAA	0.381	1.18	0.662	0.416
AAA	**0.482**	**0.527**	**10.1**	**0.00148**

SNP_A-4225817, SNP_A-1992707, SNP_A-1911590

The LD of the associated region with the haploblocks is shown in figure 6.17.

Figure 6. 17. *LD of the associated region on chromosome 8.*



For the marker on chromosome 5, the association with one haplotype was stronger than that for the individual marker (Table 6.24).

Table 6. 24. *Haplotype association analysis for the markers on chromosome region 5q23.2.*

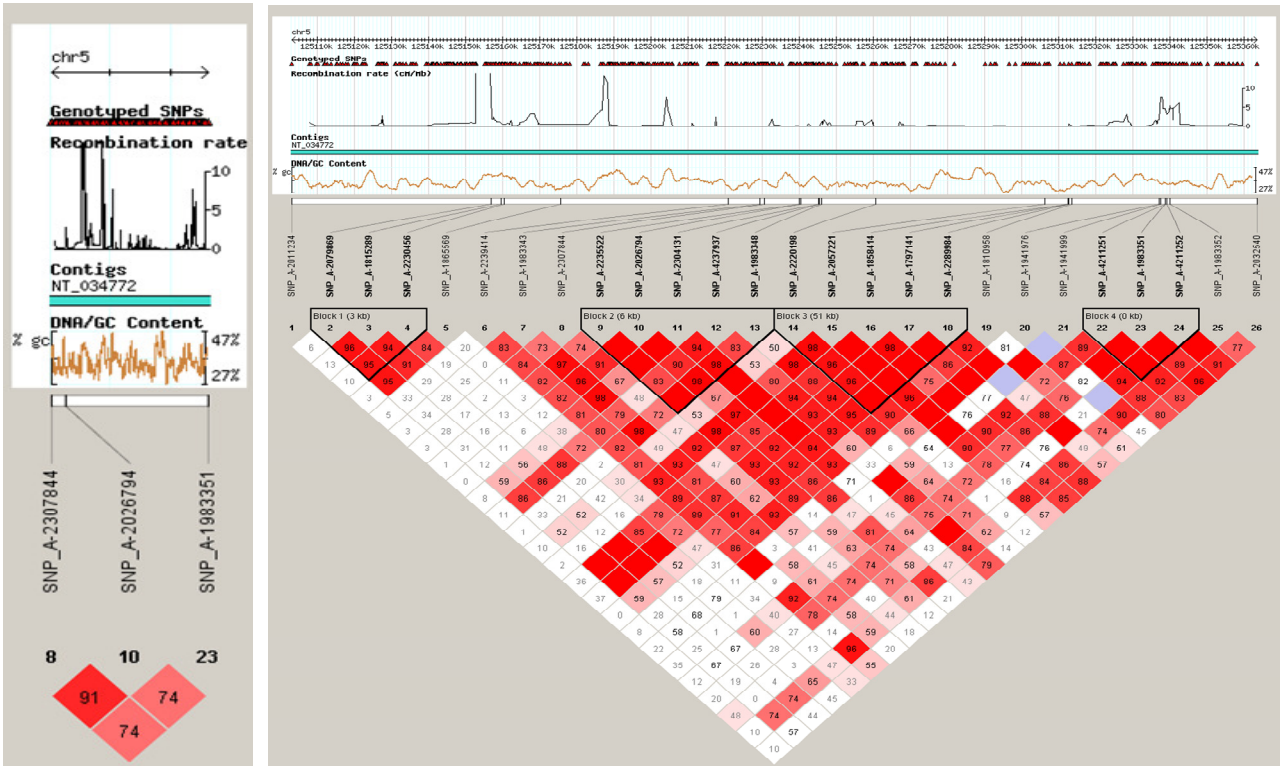
SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	OR	CHISQ	P-VALUE
SNP_A-2307844 (rs11241841)	0.443/0.325	0.917	0	*	0.232	18.3	1.93e-005
SNP_A-2026794 (rs2568395)	0.359/0.438	0.978	9.83	0.615	0.356	11.1	8.69e-004
SNP_A-1983351 (rs326041)	0.251/0.200	0.974	108	0.262	0.412	5.9	0.0151

HAPLOTYPE *..	FREQ	OR	CHISQ	P-VALUE
BAB	0.214	0.387	6.1	0.0135
BAA	0.128	0.412	4	0.0454
BBA	0.0843	0.0102	6.8	0.00914
ABA	0.517	4.54	20.4	6.16e-006

SNP_A-2307844, SNP_A-2026794, SNP_A-1983351

These three SNPs are in an intergenic region without any genes close to them (Figure 6.18).

Figure 6. 18. *LD of the associated region on chromosome 5.*



Although we excluded those SNPs which were not in HWE (p-value of filter 0.0001), the main associated SNP on chromosome 5, cytoband q23.2, was not in HWE according to the Haploview software.

For the marker on chromosome region 7p14.1, we found one haplotype but the association was a little bit weaker than that for the individual marker (Table 6.25).

Table 6. 25. *Haplotype association analysis for the markers on chromosome region 7p14.1.*

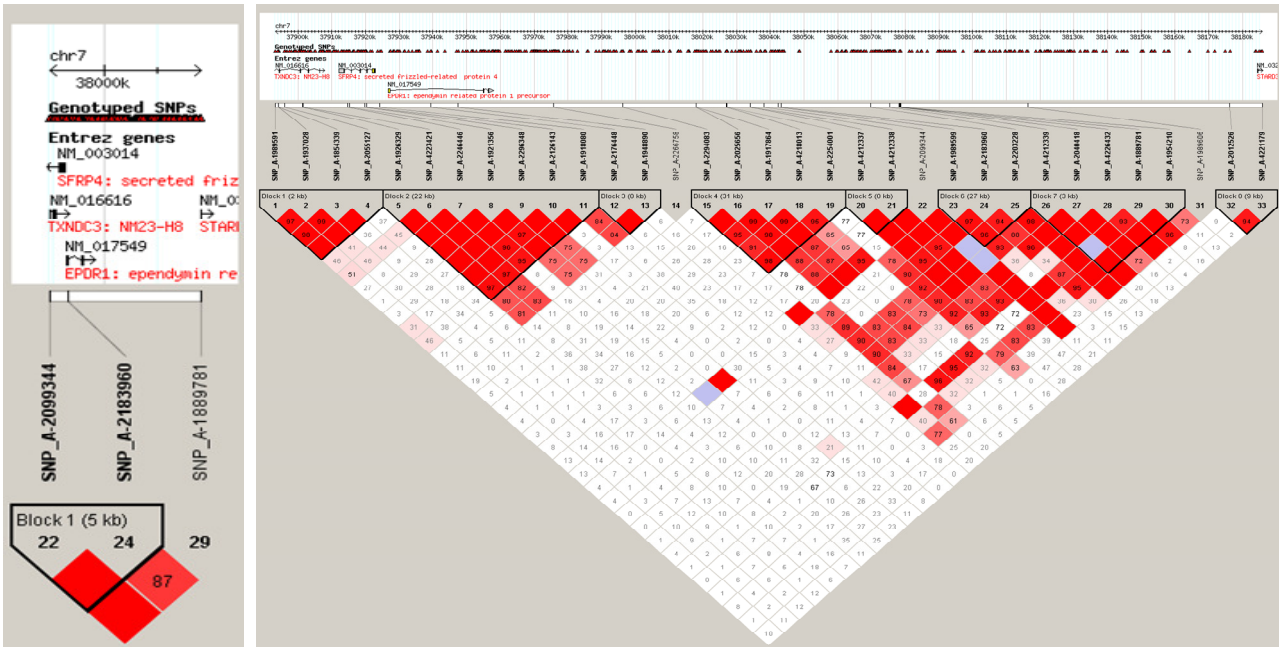
SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	OR	CHISQ	P-VALUE
SNP_A-2099344 (rs1721396)	**0.18/0.225**	**0.996**	**0**	*****	**3.18**	**18.2**	**2.04e-005**
SNP_A-2183960 (rs1721393)	0.47/0.479	0.956	5.09	0.246	1.94	6.22	0.0126
SNP_A-1889781 (rs1403986)	0.191/0.254	0.983	40.3	0.916	2.69	13.3	2.67e-004

HAPLOTYPE *..	FREQ	OR	CHISQ	P-VALUE
BBA	**0.18**	**3.1**	**17.5**	**2.8e-005**
ABB	0.288	0.843	0.395	0.53
AAB	0.519	0.525	5.88	0.0153

SNP_A-2099344, SNP_A-2183960, SNP_A-1889781

The LD of that region on chromosome 7 is shown in figure 6.19.

Figure 6. 19. *LD of the associated region on chromosome 7.*



The main marker in that region, *SNP_A-2099344* is between two haploblocks and also is a Tag SNP for *SNP_A-1889781* (R-sq=0.91). This last SNP was in the position number 62 in this association analysis.

As you can see the three SNPs are in an intergenic region close to several genes.

6.3.3.2. Association analysis for microalbuminuria

After including clinical co-variables in the model none of the SNPs reached the significance level after adjusting for multiple comparisons. Again there was not association with the marker *SNP_A-4215311* neither for the haplotype which included this SNP.

6.3.3.2.1. Models with number of drugs as co-variable

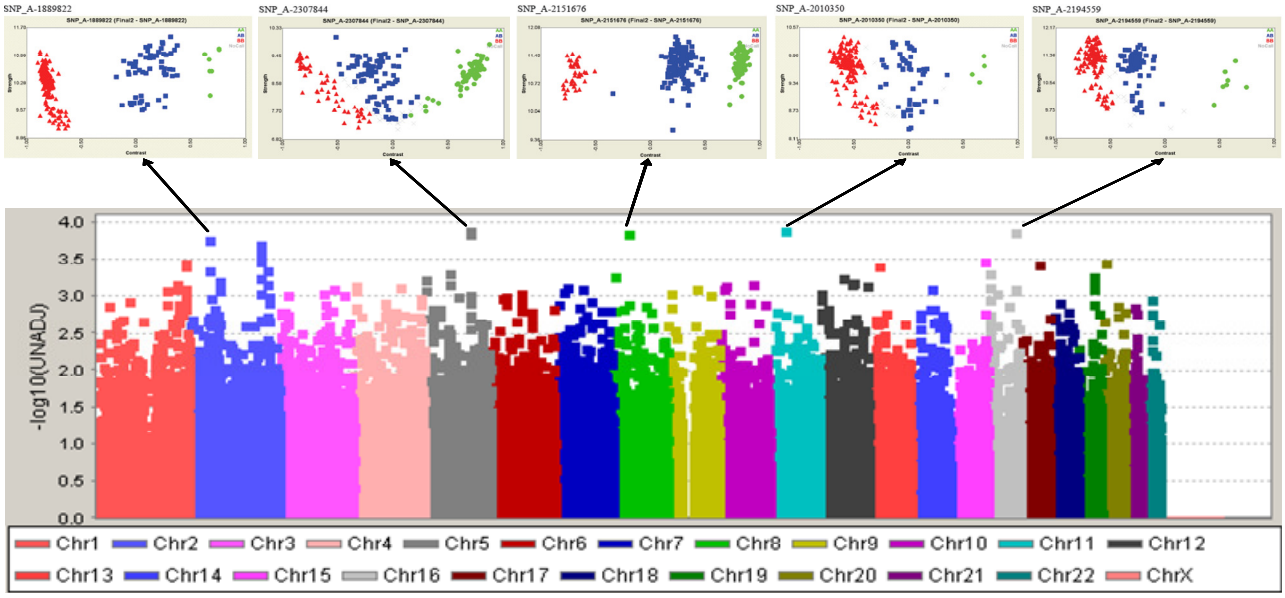
6.3.3.2.1.1. Individual SNPs analysis

The genomic control for this analysis was 1.02 with a mean chi-squared statistic of 1.00. For the models which included NºDrugs as co-variable, the most associated SNPs were far away from significance. The most associated SNPs were on chromosome regions: 11p12 [*SNP_A-2010350* (*rs355231*)], 5q23.2 [*SNP_A-2307844* (*rs11241841*) and *SNP_A-4211251* (*rs13180385*)], 16q23.1 [*SNP_A-2194559* (*rs1465099*)], 8p11.21 [*SNP_A-2151676* (*rs11787055*)], 2p16.1 [*SNP_A-1889822* (*rs10427222*)] and 2q32.2 [*SNP_A-2024873* (*rs2033870*), and *SNP_A-1844966* (*rs6719224*)], with unadjusted p-values within the range 0.00012 and 0.00030).

Many of these polymorphisms as we expected were the most associated with albuminuria including co-variables.

The Manhattan plot for this analysis is shown in figure 6.20.

Figure 6. 20. Manhattan plot for the association analysis with microalbuminuria including age, sex, BMI, SBP, glucose, eGFR (MDRD) and number of drugs as clinical co-variables.



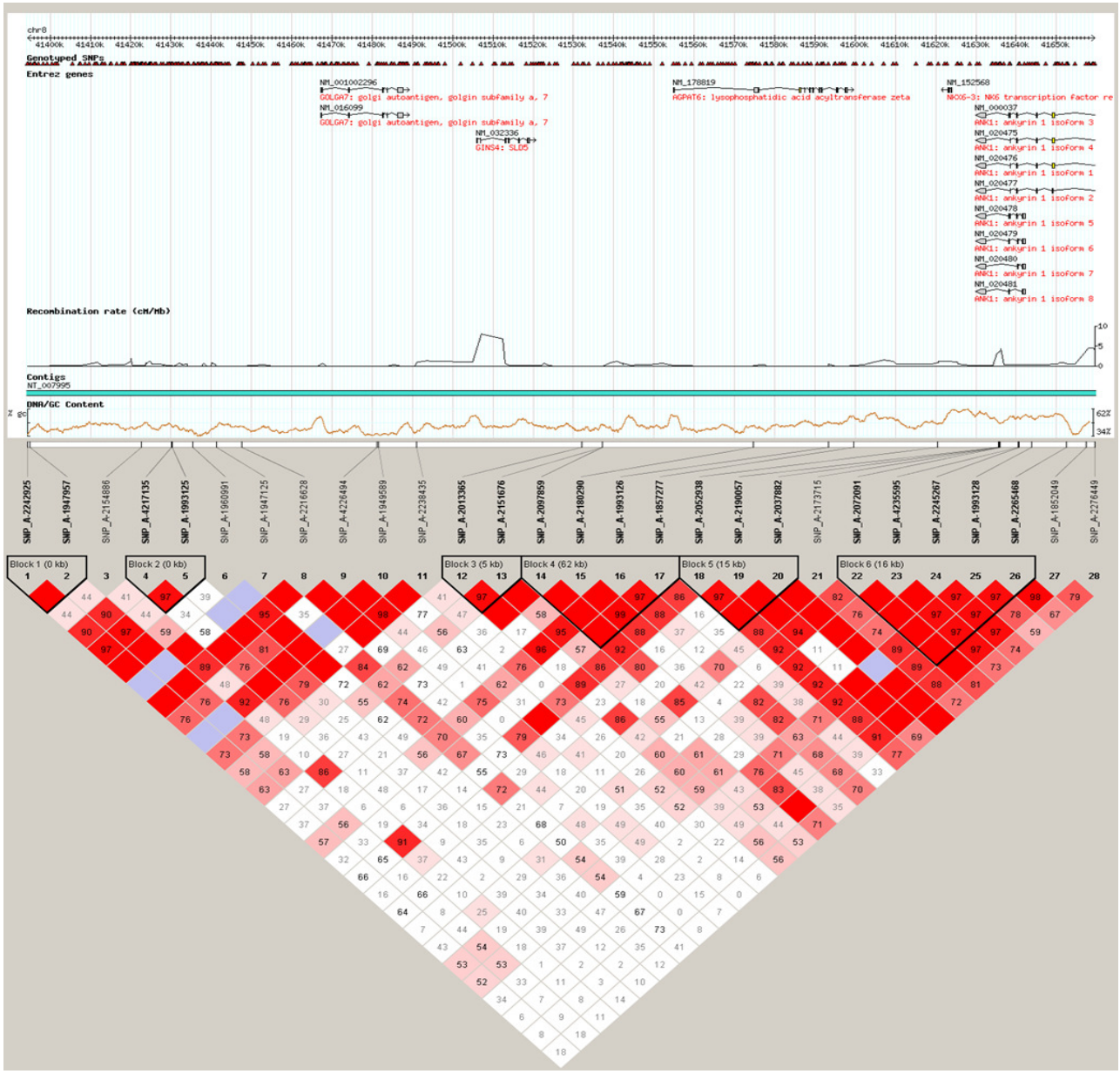
6.3.3.2.1.2. Haplotype and LD analysis

The haplotype analysis for markers on chromosome regions 11p12 and 5q23.2 are shown in tables 6.26 and 6.27. For the markers on chromosome 16q23.1, 8p11.21 and 2p16.1 we did not find any haplotype significantly associated.

The LD of some of those regions has been previously shown (Figures 6.14, 6.15 and 6.18).

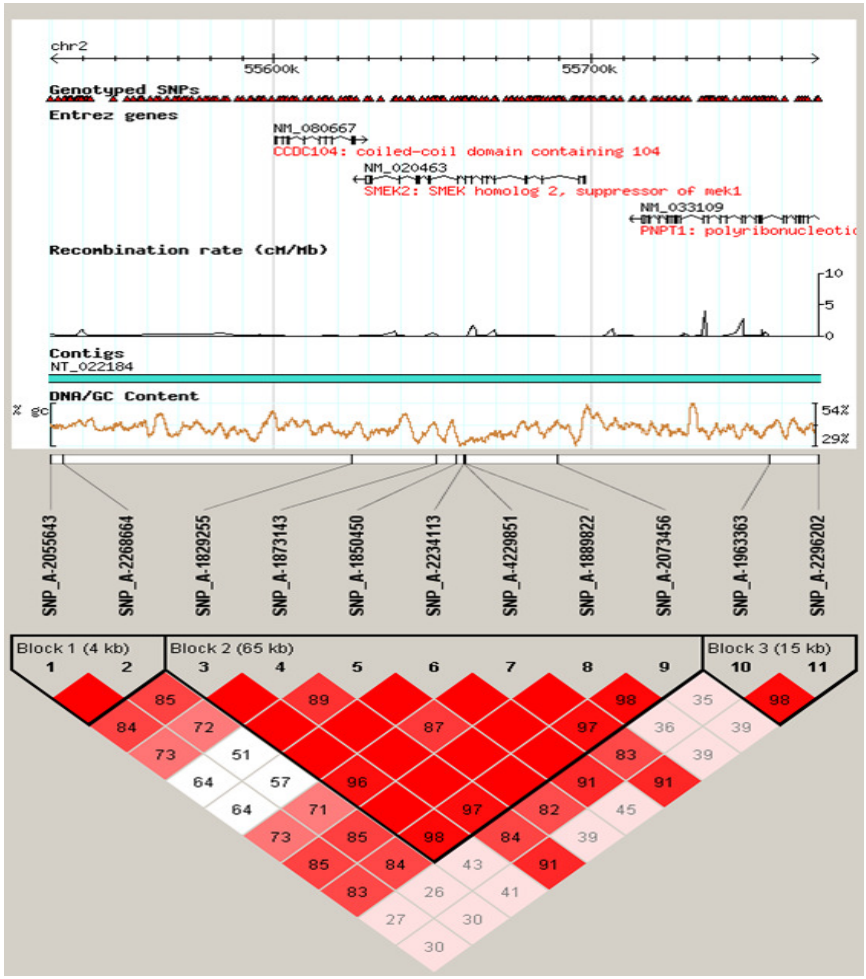
In figures 6.21 and 6.22 we show the LD of the main associated markers on chromosome regions 8p11.21 and 2p16.1 respectively.

Figure 6. 21. *LD of the associated region on chromosome 8.*



The most associated marker in this chromosomal region (8p11.21), *SNP_A-2151676*, is inside one haploblock. There are not other SNPs in high LD (R-sq>0.8) with it.

Figure 6. 22. *LD of the associated region on chromosome 2.*



The most associated marker in this region 2p16.1, *SNP_A-1889822*, belongs to one haploblock and it is in high LD wit *SNP_A-1829255* which is a tag SNP also for *SNP_A-2073456*.

The proxy association analysis for the associated markers at locus 11p12 and 5q23.2 is shown in table 6.26 and table 6.27.

Table 6. 26. *Haplotype association analysis for microalbuminuria and markers on chromosome region 11p12 including age, sex, BMI, SBP, glucose, eGFR (MDRD) and number of drugs as clinical co-variables.*

SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	OR	CHISQ	P-VALUE
SNP_A-1784634 (rs7929217)	0.15/0.117	0.968	-103	0.304	2.19	5.05	0.0246
SNP_A-2010350 (rs355231)	**0.133/0.083**	**0.973**	**0**	*	**2.82**	**8.78**	**0.00304**
SNP_A-2296471 (rs996262)	0.285/0.316	0.977	79.1	0.339	1.93	5.82	0.0159

HAPLOTYPE .*.	FREQ	OR	CHISQ	P-VALUE
BAB	**0.0811**	**2.81**	**7.06**	**0.00789**
ABB	0.146	1.32	0.632	0.427
ABA	0.658	0.508	4.25	0.0393

SNP_A-1784634, **SNP_A-2010350**, SNP_A-2296471

Table 6. 27. *Haplotype association analysis for microalbuminuria and markers on chromosome region 5q23.2 including age, sex, BMI, SBP, glucose, eGFR (MDRD) and number of drugs as clinical co-variables.*

SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	OR	CHISQ	P-VALUE
SNP_A-2307844 (rs11241841)	**0.443/0.325**	**0.914**	**0**	*	**0.205**	**19.6**	**9.65e-006**
SNP_A-2026794 (rs2568395)	0.354/0.438	0.977	9.83	0.612	0.29	14	1.8e-004
SNP_A-1983351 (rs326041)	0.249/0.200	0.982	108	0.254	0.348	7.47	0.00628

HAPLOTYPE *..	FREQ	OR	CHISQ	P-VALUE
BAB	0.211	0.304	8.3	0.00396
BAA	0.127	0.431	3.61	0.0576
BBA	0.0874	0.00968	6.41	0.0113
ABA	**0.518**	**5.22**	**22.2**	**2.45e-006**

SNP_A-2307844, SNP_A-2026794, SNP_A-1983351

Regarding to the haplotype in region q32.2 of chromosome 2 which I described in the association with albuminuria adjusted for age, sex, BMI, SBP, glucose, eGFR (MDRD) and number of drugs,

for the case of microalbuminuria the association was a little bit weaker than the previous one (Table 6.28).

Table 6. 28. *Haplotype association analysis for microalbuminuria and markers on chromosome region 2q32.2 including age, sex, BMI, SBP, glucose, eGFR (MDRD) and number of drugs as clinical co-variables.*

SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	OR	CHISQ	P-VALUE
SNP_A-2180122 (rs4667295)	0.236/0.200	0.986	-130	0.429	0.481	4.83	0.0279
SNP_A-2291588 (rs1550388)	0.201/0.146	1	-30.5	1	0.17	15.2	9.87e-005
SNP_A-1844966 (rs6719224)	**0.197/0.225**	**0.964**	**0**	*****	**0.17**	**15.2**	**9.87e-005**

HAPLOTYPE ..*	FREQ	OR	CHISQ	P-VALUE
BAB	**0.159**	**0.157**	**13.5**	**2.34e-004**
BBA	0.0769	1.62	1.37	0.241
ABA	0.722	2.2	5.28	0.0215

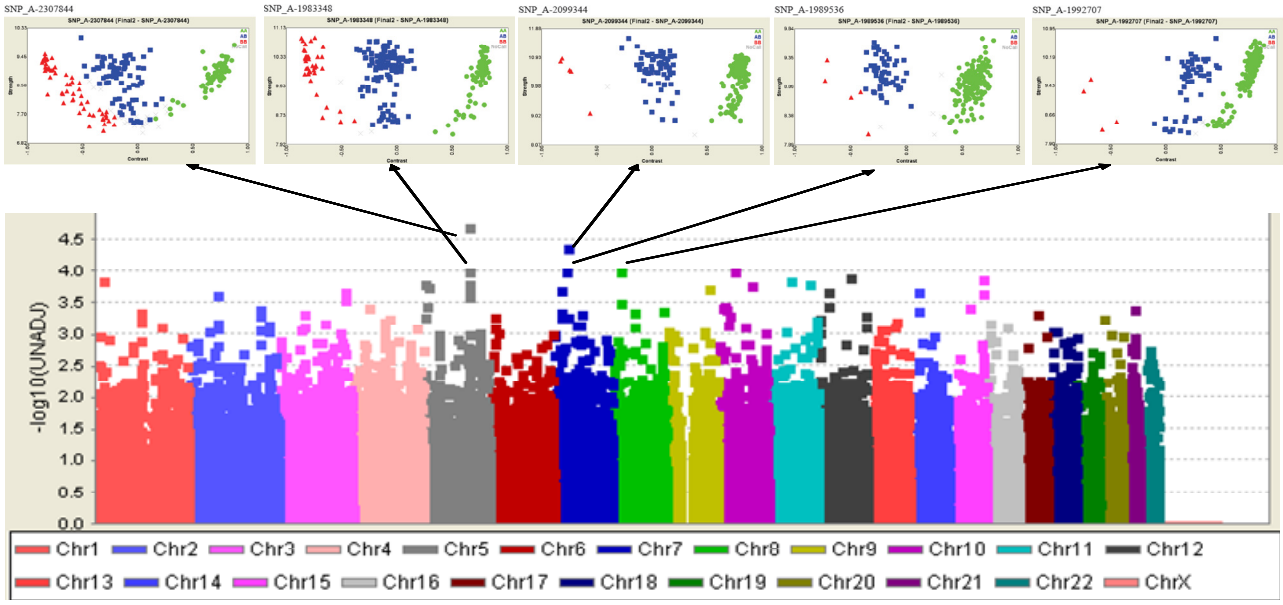
SNP_A-2180122, SNP_A-2291588, **SNP_A-1844966**

6.3.3.2.2. Models without number of drugs as co-variable

6.3.3.2.2.1. Individual SNPs analysis

For the model without including the number of drugs as co-variable, two of the most associated SNP, first [*SNP_A-2307844* (*rs11241841*)] and fourth [*SNP_A-1983348* (*rs327866*)] positions, were on chromosome 5, cytoband q23.2. The second [*SNP_A-2099344* (*rs1721396*)] and third [*SNP_A-1989536* (*rs7778558*)] most associated SNPs were on chromosome region 7p14.1 and 7p14.2 respectively. Interestingly within the region 7p14.2 is located the ELMO gene which has been previously associated with diabetic nephropathy. The most associated marker in the case of the association for albuminuria adjusted for clinical covariates excluding NºDrugs on chromosome 8, cytoband p21.2 (*SNP_A-1992707*) was in the fifth position (p-value unadjusted 9.62e-005).
The Manhattan plot for this analysis is shown in figure 6.23.

Figure 6. 23. *Manhattan plot for the association analysis with microalbuminuria including age, sex, BMI, SBP, glucose, and eGFR (MDRD) as clinical co-variables.*



6.3.3.2.2.2. Haplotype and LD analysis

As I said before, the LD of some of the associated regions has been previously described Regarding to the haplotype analysis we found stronger association with one haplotype which include the markers on chromosome 5 than that observed for the individual SNPs (Table 6.29).

Table 6. 29. *Haplotype association analysis for microalbuminuria and markers on chromosome region 5q23.2 including age, sex, BMI, SBP, glucose and eGFR (MDRD) as clinical co-variables.*

SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	OR	CHISQ	P-VALUE
SNP_A-2307844 (rs11241841)	**0.443/0.325**	**0.914**	**0**	*	**0.205**	**19.7**	**8.91e-006**
SNP_A-1983348 (rs327866)	0.406/0.375	0.991	15.5	0.459	0.33	14.4	1.46e-004
SNP_A-2057221 (rs7727284)	0.262/0.208	0.999	75.6	0.415	0.328	9.15	0.00248

HAPLOTYPE *..	FREQ	OR	CHISQ	P-VALUE
BBB	0.252	0.346	8.08	0.00447
BBA	0.0905	0.335	3.48	0.0619
ABA	0.0575	0.935	0.0188	0.891
BAA	0.0896	0.0253	5.7	0.017
AAA	**0.5**	**4.36**	**22.6**	**1.96e-006**

SNP_A-2307844, SNP_A-1983348, SNP_A-2057221

We also found a stronger association with another haplotype including the markers on chromosome 7 (Table 6.30).

Table 6. 30. *Haplotype association analysis for microalbuminuria and markers on chromosome region 7p14.1 including age, sex, BMI, SBP, glucose and eGFR (MDRD) as clinical co-variables.*

SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	OR	CHISQ	P-VALUE
SNP_A-2266758 (rs1668349)	0.409/0.350	0.991	-62.5	0.145	0.578	19.7	0.447
SNP_A-2099344 (rs1721396)	**0.173/0.225**	**0.995**	**0**	*****	**18.1**	**14.4**	**2.05e-005**
SNP_A-1989606 (rs10280461)	0.491/0.295	0.995	78.2	0.201	1.13	9.15	0.288

HAPLOTYPE .*.	FREQ	OR	CHISQ	P-VALUE
AAA	0.363	0.731	1.18	0.278
BAA	0.127	1.14	0.139	0.709
BBB	**0.174**	**3.42**	**20.4**	**6.34e-006**
BAB	0.291	0.397	6.52	0.0107
AAA	**0.5**	**4.36**	**22.6**	**1.96e-006**

SNP_A-2266758, **SNP_A-2099344**, SNP_A-1989606

For the marker on chromosome 7, cytoband p14.2, the association with the haplotype was weaker than that for the individual analysis (Table 6.31).

Table 6. 31. *Haplotype association analysis for microalbuminuria and markers on chromosome region 7p14.2 including age, sex, BMI, SBP, glucose and eGFR (MDRD) as clinical co-variables.*

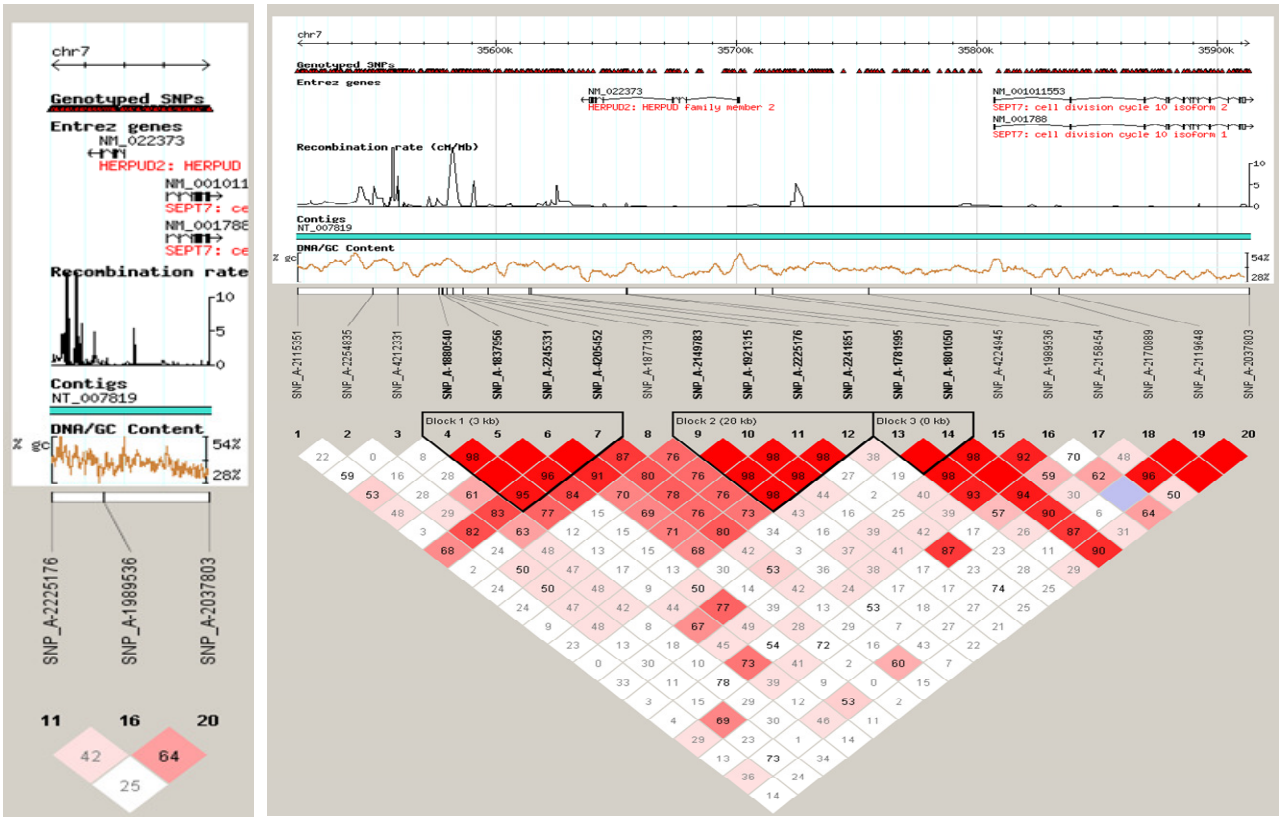
SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	OR	CHISQ	P-VALUE
SNP_A-2225176 (rs4723436)	0.234/0.208	0.995	-101	0.12	1.21	0.423	0.515
SNP_A-1989536 (rs7778558)	**0.144/0.125**	**0.986**	**0**	*****	**3.41**	**16.7**	**4.45e-005**
SNP_A-2037803 (rs10233082)	0.326/0.308	0.973	198	0.14	1.75	4.06	0.044

HAPLOTYPE .*.	FREQ	OR	CHISQ	P-VALUE
BBA	0.0536	1.72	1.81	0.177
ABA	**0.0552**	**3.19**	**7.01**	**0.00809**
AAA	0.212	1.36	1	0.316
BAB	0.143	0.418	3.56	0.059
AAB	0.495	0.732	1.25	0.263

SNP_A-2225176, **SNP_A-1989536**, SNP_A-2037803

The LD of that region on chromosome 7 is shown in figure 6.24.

Figure 6. 24. *LD of the associated region on chromosome 7.*



The main associated marker is not in high LD (R-sq>0.8) with others markers.

The association with a similar haplotype than that previously described for albuminuria with co-variables on chromosome region 8p21.2 was stronger for the case of microalbuminuria (Table 6.32).

Table 6. 32. *Haplotype association analysis for microalbuminuria and markers on chromosome region 8p21.2 including age, sex, BMI, SBP, glucose and eGFR (MDRD) as clinical co-variables.*

SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	OR	CHISQ	P-VALUE
SNP_A-1924618 (rs9314285)	0.148/0.258	0.946	-80.8	0.721	2.42	8.19	0.00422
SNP_A-4225817 (rs12551522)	0.385/0.30	0.982	-64	0.0838	0.929	0.0725	0.788
SNP_A-1992707 (rs4872244)	**0.119/0.229**	**0.968**	**0**	*****	**3.16**	**11.3**	**7.78e-004**

HAPLOTYPE ..*	FREQ	OR	CHISQ	P-VALUE
BAB	**0.115**	**3.14**	**11.6**	**6.74e-004**
ABA	0.355	0.941	0.0493	0.824
AAA	0.493	0.586	4.11	0.0427

SNP_A-1924618, **SNP_A-4225817**, SNP_A-1992707

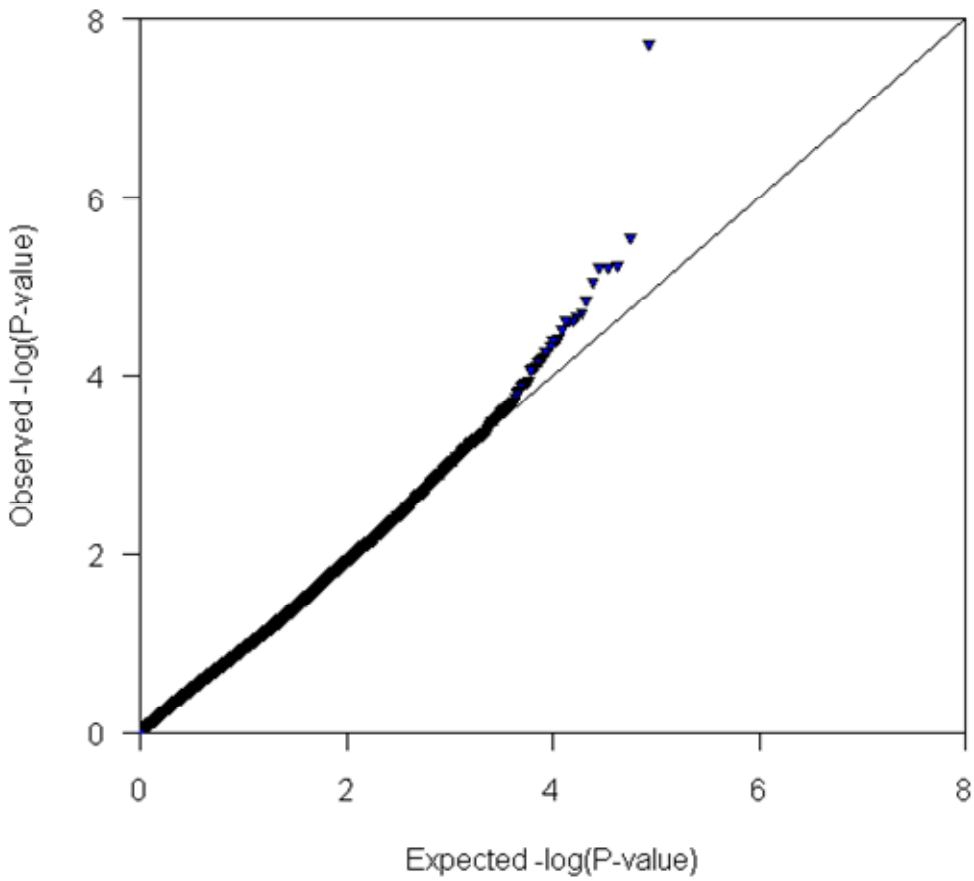
Also two of the SNP of this haplotype are in intronic region of the ADAM7 gene.

6.3.4. Association analysis for quantitative traits (Unadjusted)

6.3.4.1. Association analysis for UAE

The genomic control for this analysis was 1.07 with a mean chi-square of 1.02. Below is shown the Q-Q plot for this analysis (Figure 6.25) and the graphical representation of the results (Figure 6.26).

Figure 6. 25. *Q-Q plot for the observed and expected p-values (adjusted for GC) for the association analysis with UAE.*



6.3.4.1.1. Individual SNPs analysis

In the analysis for the quantitative trait, we found two SNPs which reached the statistical significance even after Bonferroni correction.

The most associated one was also located on chromosome 8 and correspond to the marker, *SNP_A-4205634* (*rs41534844*) (p-value unadjusted=1.17×10^{-9}, Bonferroni p-value=0.00019). The empirical p-value for the adaptative permutation analysis was 7e-006 after one million of

permutations. This marker, in contrast to the one associated with the qualitative trait, is located not in the short arm but in the long arm of chromosome 8 (cytoband q12.1). Carriers of the minor allele of this SNP had higher levels of UAE than carriers of the major allele (Beta 49.8 (34.6-65.1), p-value 1.17×10^{-9}).

This marker, *rs41534844*, is very close (less than 100Kb) to a non coding RNA gene (U6 spliceosomal RNA) and to the inositol monophosphatase domain containing 1 gene (IMPAD1) and there were not other SNPs in high LD (R-sq>0.8) with it.

The other marker which was significantly associated with UAE was on chromosome 2 (cytoband p11.2) and corresponded to the marker, *SNP_A-2207401* (*rs11675985*) (p-value unadjusted=6.39×10^{-9}, Bonferroni p-value=0.001). The empirical p-value for the adaptative permutation analysis was 1.0e-005 after one million of permutations. This SNP is located in an intronic region of the Receptor Expression-Enhancing Protein 1 (REEP1) gene and it is not in high LD with another markers.

The third most associated marker although it was not statistically significant was on chromosome 9 [*SNP_A-2169005* (*rs1339550*) (p-value unadjusted=1.30e-006, Bonferroni p-value=0.220)]. This SNP is located in cytoband p22.2, in an intronic region of the basonuclin (BNC2) gene.

None of the SNP previously associated with the qualitative trait reached the statistical significance.

Figure 6. 26. *Manhattan plot for the association analysis with UAE.*

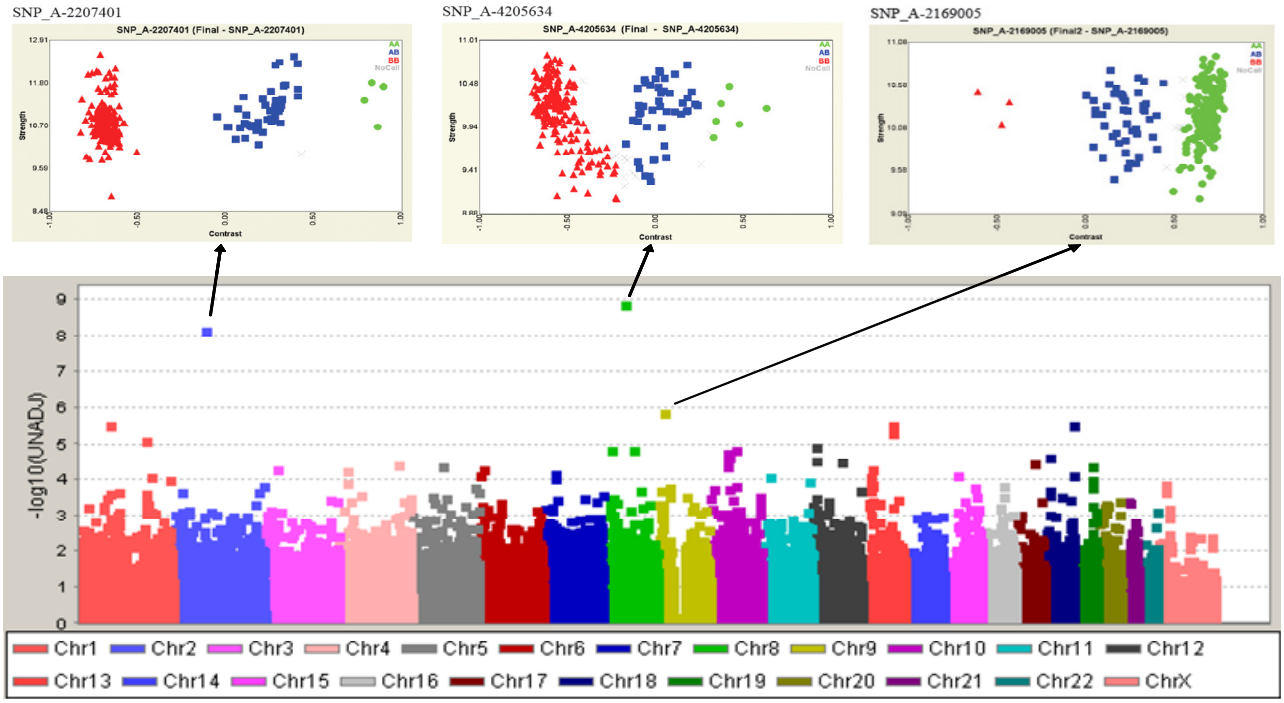


Table 6. 33. *Characteristics of the main associated SNPs with UAE.*

SNPID	Call Rate	%AA	%AB	%BB	MAF	H.W. p-Value	dbSNP rs ID	Chr	Physical Position	Allele A	Allele B
SNP_A-4205634	94.74	2.63	18.42	73.68	0.125	0.102	rs41534844	8	57985421	C	T
SNP_A-2207401	99.56	1.75	18.42	79.38	0.110	0.398	rs11675985	2	86412486	G	T
SNP_A-2169005	96.93	75.87	19.73	1.31	0.115	0.969	rs1339550	9	16844839	C	T

.

Table 6. 34. *Linear regression for the main associated SNPs with UAE.*

Chr	SNP	BP	A1	BETA	SE	UL	LL	STAT	p-value
8q12.1	SNP_A-4205634 (rs41534844)	57985421	A	49.87	7.78	34.6	65.1	6.41	1.178e-009
2p11.2	SNP_A-2207401 (rs11675985)	86412486	A	54.9	9.05	37.21	72.56	6.09	6.396e-009
9p22.2	SNP_A-2169005 (rs1339550)	16844839	B	44.08	8.80	26.83	61.34	5.07	1.301e-006

A1: minor allele; UL: upper limit; LL: lower limit.

6.3.4.1.2. Haplotype and LD analysis

We also found one haplotype including the most associated marker significantly associated although the level of association was weaker than that for the individual marker (Table 6.35, Figure 6.27).

The same happened for the second most associated SNP on chromosome 2 (Table 6.36, Figure 6.28).

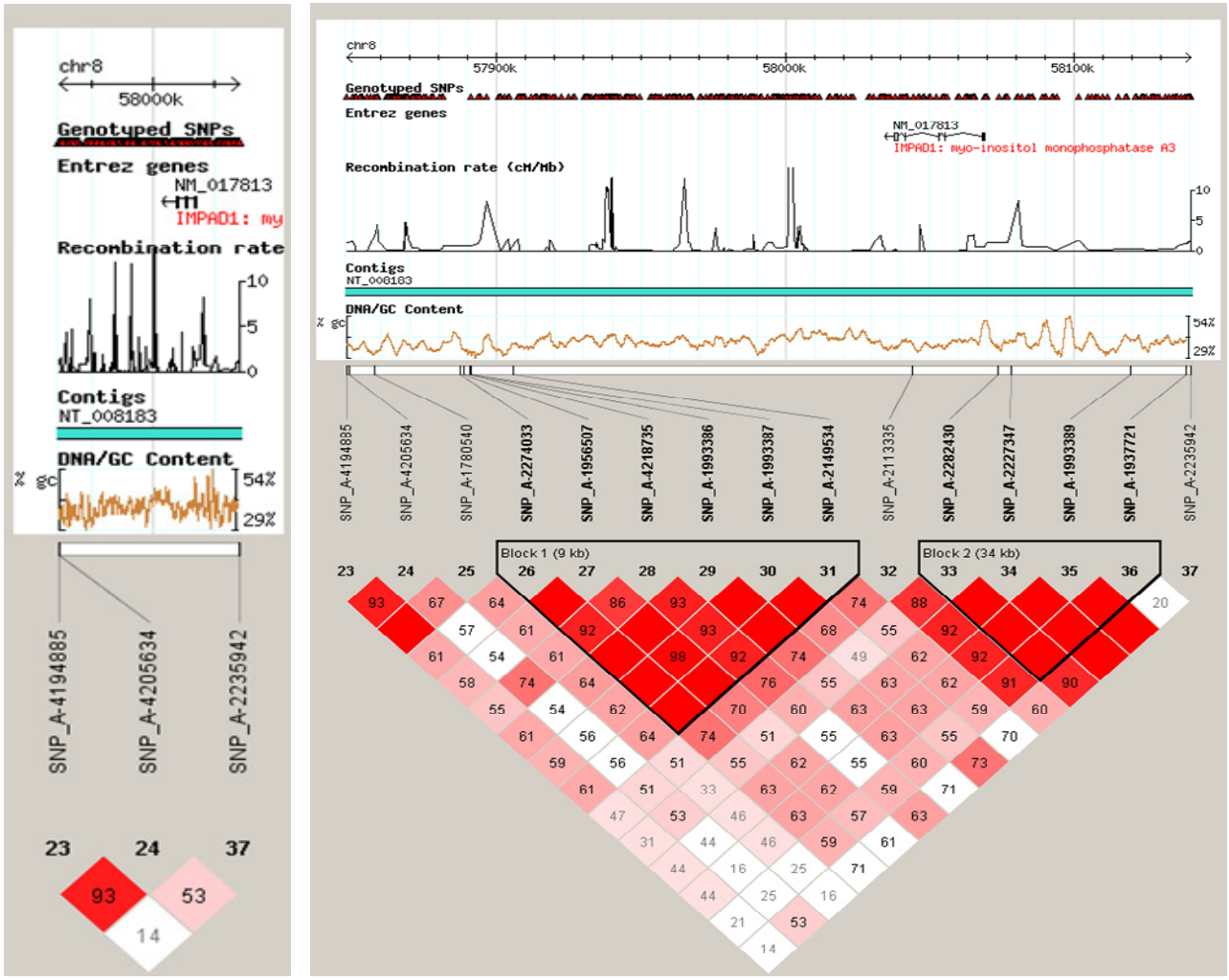
Table 6. 35. *Haplotype association analysis for the markers on chromosome region 8q12.1 and UAE.*

SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	BETA	STAT	P-VALUE
SNP_A-4194885 (rs985942)	0.495/0.460	0.939	-0.62	0.122	13.4	2.43	0.016
SNP_A-4205634 (rs41534844)	**0.119/0.100**	**0.952**	**0**	*****	**48.7**	**6.37**	**1.43e-009**
SNP_A-2235942 (rs11992681)	0.321/0.	0.978	155	0.0887	13.5	2.08	0.0384

HAPLOTYPE .*.	FREQ	BETA	STAT	P-VALUE
AAB	**0.0847**	**62.2**	**6.05**	**8.04e-009**
ABB	0.104	-9.4	-0.848	0.397
BBB	0.134	-9.63	-0.98	0.328
ABA	0.277	-6.97	-1.06	0.291
BBA	0.363	-13.9	-2.23	0.0268

SNP_A-4194885, **SNP_A-4205634**, SNP_A-2235942

Figure 6. 27. *LD of the associated region on chromosome 8.*



As you can see the main associated marker is in LD with *SNP_A-4194885* (R-sq = 0.114, D' = 0.933).

Table 6. 36. *Haplotype association analysis for the markers on chromosome region 2p11.2 and UAE.*

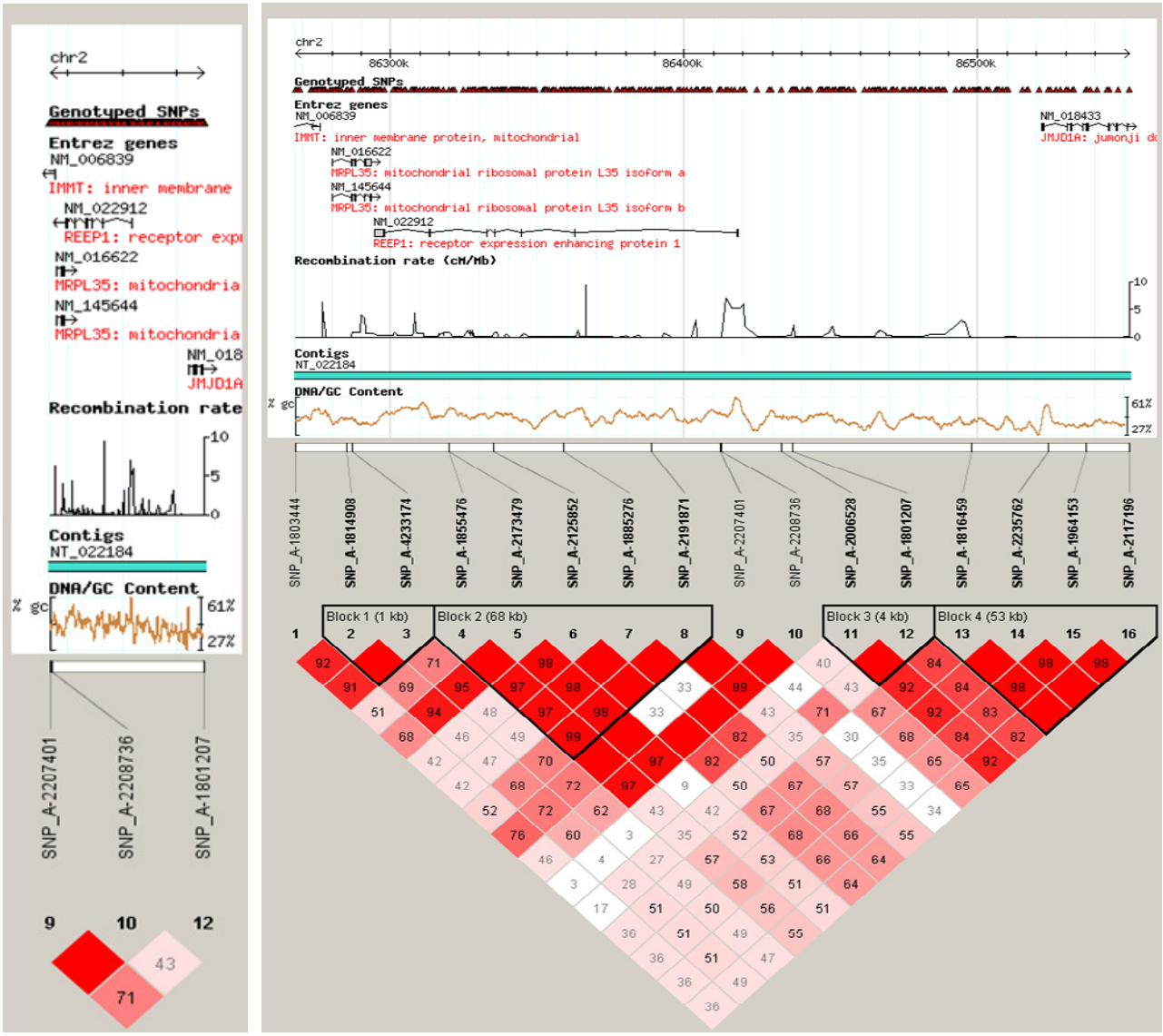
SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	BETA	STAT	P-VALUE
SNP_A-2207401 (rs11675985)	**0.1/0.083**	**0.999**	**0**	*****	**54.9**	**6.09**	**6.4e-009**
SNP_A-2208736 (rs1863052)	0.469/0.429	0.999	0.383	0.0988	-0.911	-0.159	0.874
SNP_A-1801207 (rs12714182)	0.443/0.408	0.999	24.7	0.0725	11.3	2.01	0.0459

HAPLOTYPE *..	FREQ	BETA	STAT	P-VALUE
BBA	0.313	-3.81	-0.622	0.535
AAA	**0.0847**	**57.2**	**5.92**	**1.52e-008**
BBB	0.156	4.65	0.587	0.558
BAB	0.385	-17	-2.89	0.00425

SNP_A-2207401, SNP_A-2208736, SNP_A-1801207

Two of the markers included in this haplotype are in intronic regions of the REEP1 gene and the other is located in a gene which codifies the U8 small nucleolar RNA and therefore can regulate the gene expression.

Figure 6. 28. *LD of the associated region on chromosome 2.*



The most associated marker is not in high LD (r-sq>0.8) with others markers. The *SNP_A-2208736* is a Tag SNP for: *SNP_A-2191871* and *SNP_A-1855476*.

For the marker on chromosome 9, the haplotype analysis was not conclusive (Table 6.37).

Table 6. 37. *Haplotype association analysis for the markers on chromosome region 9p22.2 and UAE.*

SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	BETA	STAT	P-VALUE
SNP_A-2169005 (rs1339550)	0.117/0.150	0.987	0	*	44	5.03	1.13e-006
SNP_A-2211656 (rs3927680)	0.465/0.354	0.991	32.5	0.154	11.6	1.91	0.0571
SNP_A-1804453 (rs10810668)	0.205/0.200	0.991	57.6	0.114	15.	2.02	0.0443

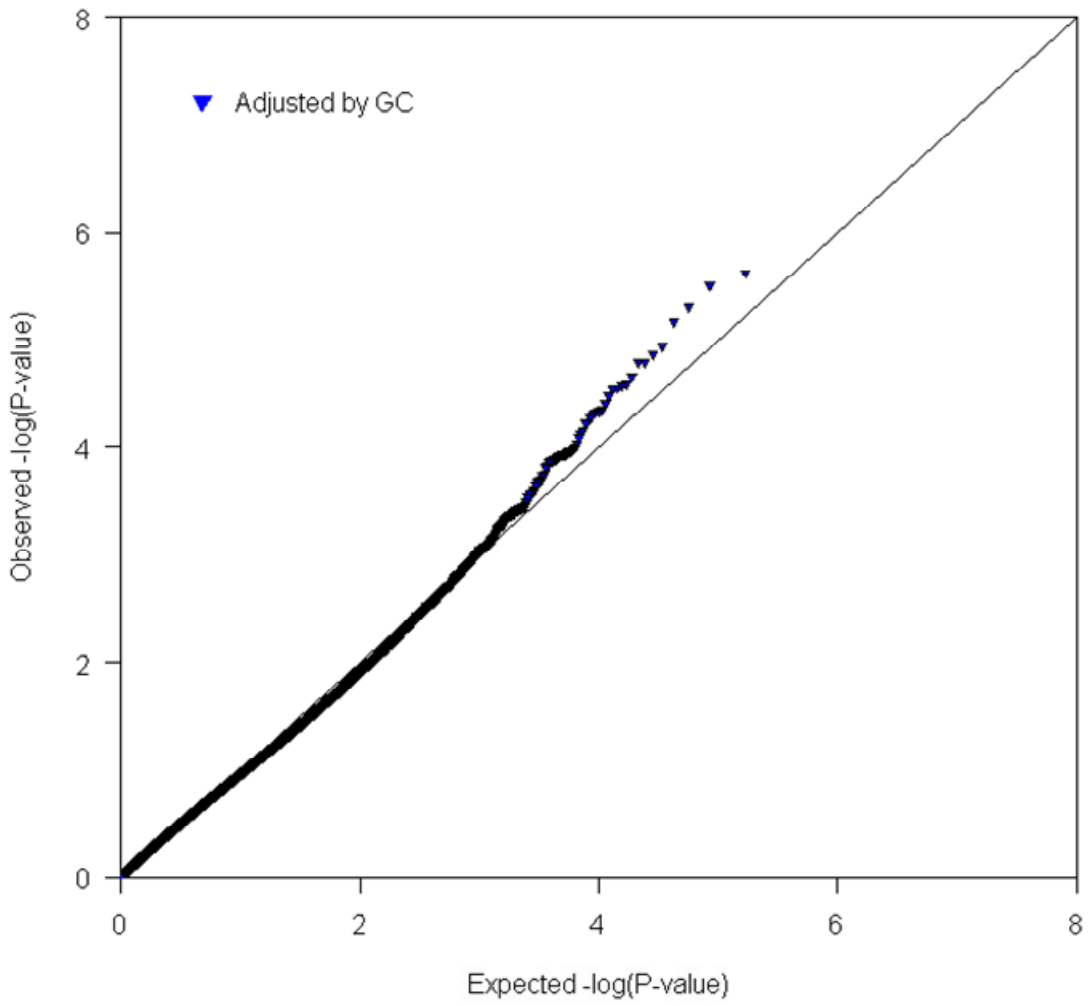
HAPLOTYPE *..	FREQ	BETA	STAT	P-VALUE
BAB	0.068	52.1	4.53	1.04e-005
AAB	0.122	-10.2	-1.03	0.302
AAA	0.225	-5.17	-0.694	0.489
ABA	0.521	-12.5	-2.04	0.0426

SNP_A-2169005, SNP_A-2211656, SNP_A-1804453

6.3.4.2. Association analysis for UAE after excluding patients with macroalbuminuria

The genomic control for this analysis was 1.05 with a mean chi-square statistic of 1.01.

Figure 6.29. *Q-Q plot for the association analysis with UAE excluding patients with macroalbuminuria (UAE≥300mg/day).*



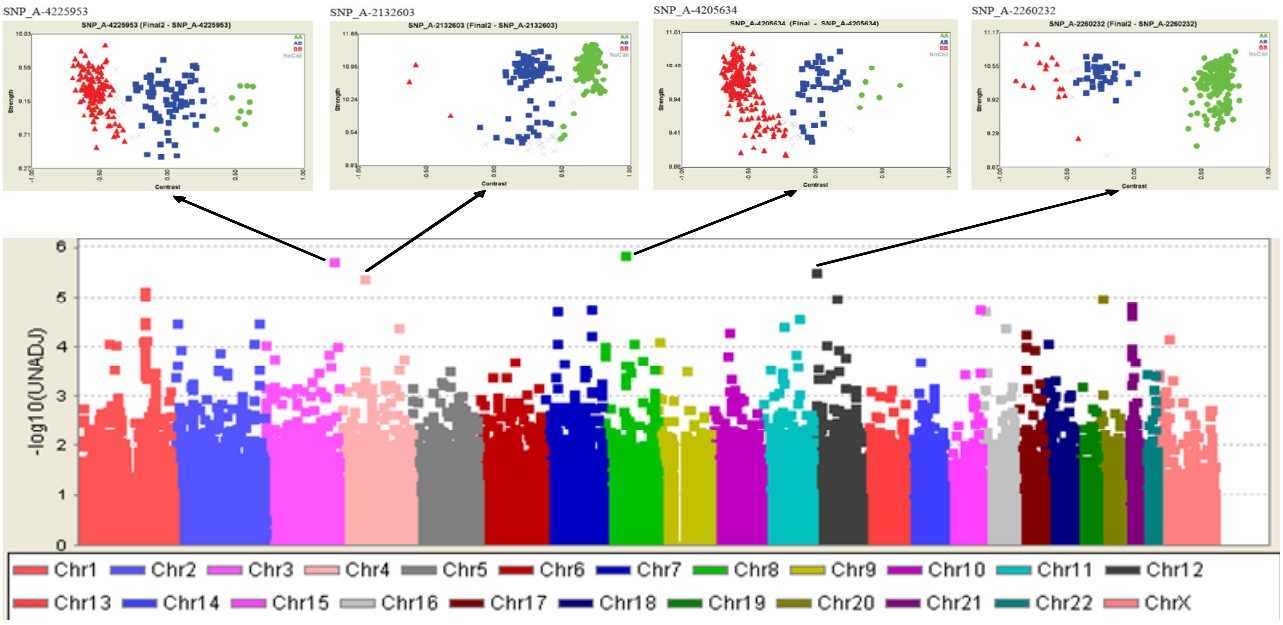
6.3.4.2.1. Individual SNPs analysis

In this case the most associated SNP was also on chromosome 8 (cytoband q12.1), *SNP_A-4205634* (*rs41534844*), but it was not statistically significance after Bonferroni correction (unadjusted p-value=1.34e-006, Bonferroni p-value=0.227). There was not association with the previously associated marker on chromosome 2, *SNP_A-2207401* (*rs11675985*).

The second most associated marker, *SNP_A-4225953* (*rs4859184*), was on chromosome region 3q27.1, in an intronic region of the MCF2 cell line derived transforming sequence-like 2 (MCF2L2) gene. The third marker was on chromosome 12 cytoband p13.2, *SNP_A-2260232* (*rs17808125*), in an intronic region of the 12 open reading frame 59 (C12orf59) gene and a few kb from the Oxidized low-density lipoprotein receptor 1 (OLR1) gene. The fourth marker was on chromosome 4q13.1in an intronic region of the EPH receptor A5 (EPHA5) gene which belongs to the protein-tyrosine kinase family and it is involved in the intracellular signalling.

The Manhattan plot for this analysis is shown in figure 6.30.

Figure 6.30. *Manhattan plot for the association analysis with UAE excluding subjects with macroalbuminuria (UAE≥300mg/day).*



6.3.4.2.2. Haplotype and LD analysis

Interestingly the association with the haplotype, *AAB*, on chromosome 8 which was previously described in the association analysis for UAE including also macroalbuminurics was significantly stronger than the association with the individual marker (*SNP_A-4205634*) (Table 6.38).

Table 6. 38. *Haplotype association analysis for the markers on chromosome region 8q12.1 and UAE after excluding patients with UAE≥300mg/day.*

SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	BETA	STAT	P-VALUE
SNP_A-4194885 (rs985942)	0.495/0.460	0.941	-0.627	0.116	5.42	1.58	0.116
SNP_A-4205634 (rs41534844)	**0.113/0.100**	**0.959**	**0**	*****	**25.1**	**4.95**	**1.69e-006**
SNP_A-2235942 (rs11992681)	0.319/0.	0.977	155	0.0861	9.13	2.31	0.0218

HAPLOTYPE .*.	FREQ	BETA	STAT	P-VALUE
AAB	**0.0811**	**34.7**	**5.76**	**3.49e-008**
ABB	0.108	-4.72	-0.702	0.468
BBB	0.132	-2.82	-0.468	0.641
ABA	0.276	-4.73	-1.17	0.242
BBA	0.368	-5.93	-1.53	0.127

SNP_A-4194885, **SNP_A-4205634**, SNP_A-2235942

For the marker on chromosome 3, there were not other SNPs in high LD with it and the association with haplotypes was weaker than that for the individual analysis (Table 6.39).

Table 6. 39. *Haplotype association analysis for the markers on chromosome region 3q27.1 and UAE after excluding patients with UAE≥300mg/day.*

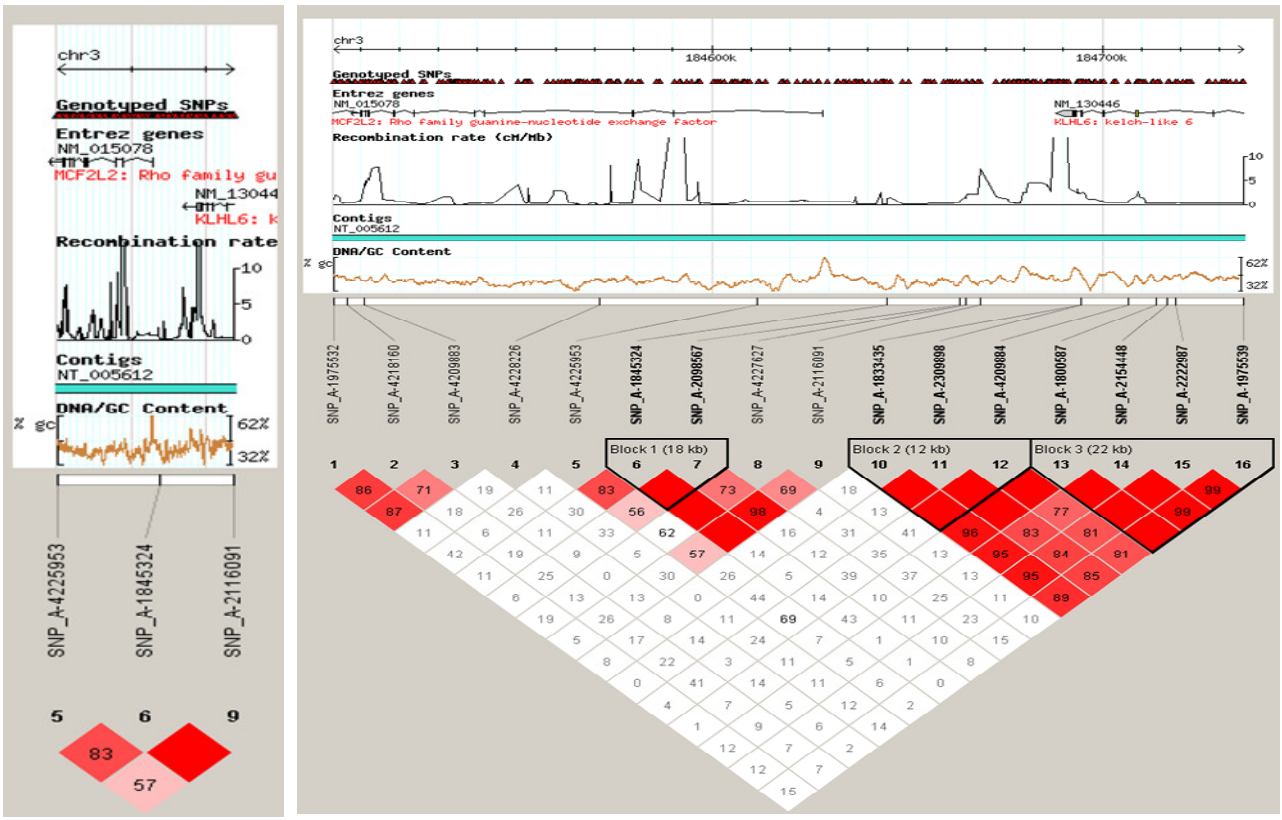
SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	BETA	STAT	P-VALUE
SNP_A-4225953 (rs4859184)	0.201/0.167	0.955	0	*	20.8	4.89	2.22e-006
SNP_A-1845324 (rs6808204)	0.181/0.143	0.986	33	0.0474	-2.86	-0.634	0.527
SNP_A-2116091 (rs1911979)	0.48/0.479	1	56.8	0.0689	-6.29	-1.85	0.0661

HAPLOTYPE *..	FREQ	BETA	STAT	P-VALUE
BBB	0.436	-8.32	-2.36	0.0193
BAA	0.178	-2.84	-0.629	0.53
ABA	0.157	21.5	4.65	6.25e-006
BBA	0.185	-5.27	-1.1	0.272

SNP_A-4225953, SNP_A-1845324, SNP_A-2116091

The LD of that region on chromosome 3 is shown in figure 6.31.

Figure 6. 31. *LD of the associated region on chromosome 3.*

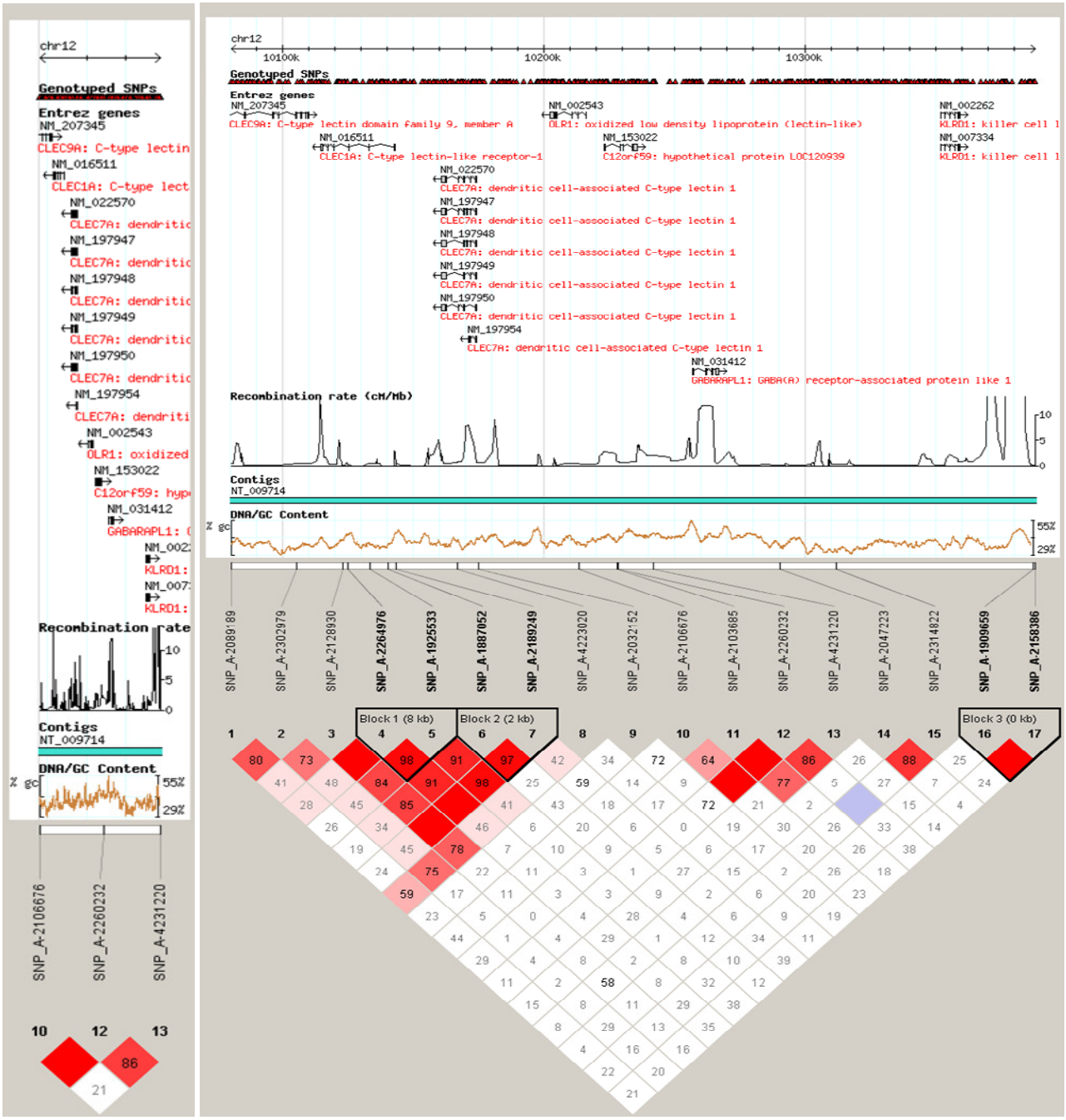


For the markers on chromosome region 12p13.2 and chromosome region 4q13.1 we did not find haplotypes clearly associated.

On chromosome 12, the most associated haplotype, *BBB*, of the following SNPs: *SNP_A-2106676*, *SNP_A-2260232*, *SNP_A-4231220*, had a p-value= 0.000375.

The LD of that region of chromosome 12 is shown in figure 6.32.

Figure 6. 32. *LD of the associated region on chromosome 12.*



On chromosome 4, the most associated haplotype forming with alleles *BBB*, of the following SNPs: *SNP_A-2132603*, *SNP_A-4203758*, *SNP_A-4198862*, had a p-value= 0.000318. The LD of that region on chromosome 4 is shown in figure 6.33.

Figure 6. 33. LD of the associated region on chromosome 4.

6.3.5. Association analysis for quantitative traits adjusted for clinical co-variables

6.3.5.1. Association analysis for UAE

The genomic control for these models was around 1.02 with a mean chi-square of around 1.00 and included 175 individuals with no missing co-variables.

6.3.5.1.1. Models with number of drugs as co-variable

6.3.5.1.1.1. Individual SNPs analysis

If we include in the model the number of anti-hypertensive drugs, the marker on chromosome 8 (*SNP_A-4205634*) remained significantly associated (second position with a p-value=1.10e-007 , Bonferroni p-value=0.01, for the model including age, sex, BMI, SBP, glucose, eGFR by MDRD and treatment). In this case the most associated marker was in the chromosome 18, *SNP_A-*

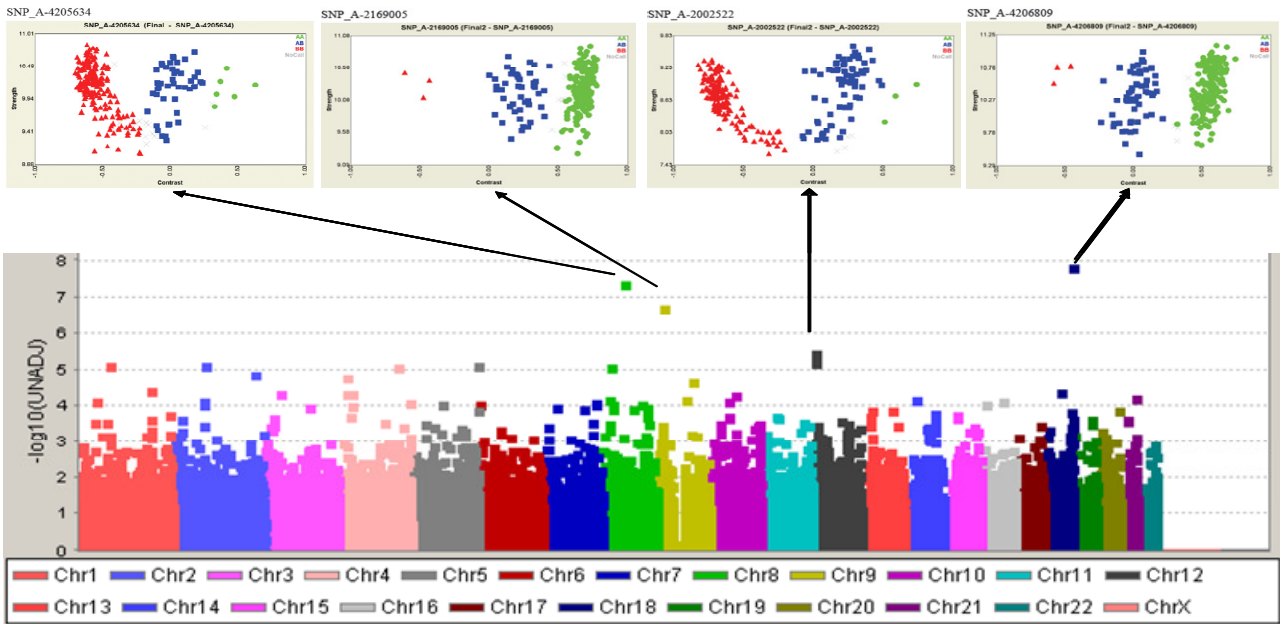
4206809 (*rs1876021*) (p-value unadjusted=5.49x10^{-8} , Bonferroni p-value=0.0093). This SNP appeared in the sixth position in the model which did not include the treatment as co-variable but it was not significantly associated (unadjusted p-value=2.59e-006, Bonferroni p-value=0.43). This marker is in the region 18q23 and there are not genes close to this marker. There are not SNP in high LD with this SNP (R-sq >0.8).

A third marker also reached the statistical significance even when considering Bonferroni correction for multiple comparisons. This marker, *SNP_A-2169005* (*rs1339550*) (unadjusted p-value=1.92e-007, Bonferroni p-value=0.032), is located in the chromosome region 9p22.2 in an intron of the basonuclin (BNC2) gene. This marker was the third associated SNP for UAE although did not reach the statistical significance neither for the haplotype (unadjusted p-value =1.3e-006, Bonferroni p-value=0.220). In the model which did not include the treatment as clinical co-variable, this SNPs was also in the third position but was not statistically significance (unadjusted p-value =6.2e-007, Bonferroni p-value=0.10).

There were also several SNPs on chromosome 12, cytoband p13.2, but they were not statistically significant. Those SNPs are in intronic regions of one gene which belongs to the killer cell lectin-like receptor subfamily K. These genes are expressed in the natural killer cells and therefore are involved in the immune response.

The Manhattan plot for this analysis is shown in figure 6.34.

Figure 6. 34. *Manhattan plot for the association analysis for UAE adjusted for age, sex, BMI, SBP, glucose, eGFR (MDRD) and number of drugs.*



6.3.5.1.1.2. Haplotype and LD analysis

On chromosome 18 we found one haplotype with similar association to the one obtained for the individual marker (Table 6.40). As you can see in figure 6.35 the marker on chromosome 18 is located between two blocks of high LD.

Figure 6. 35. *LD of the associated region on chromosome 18.*

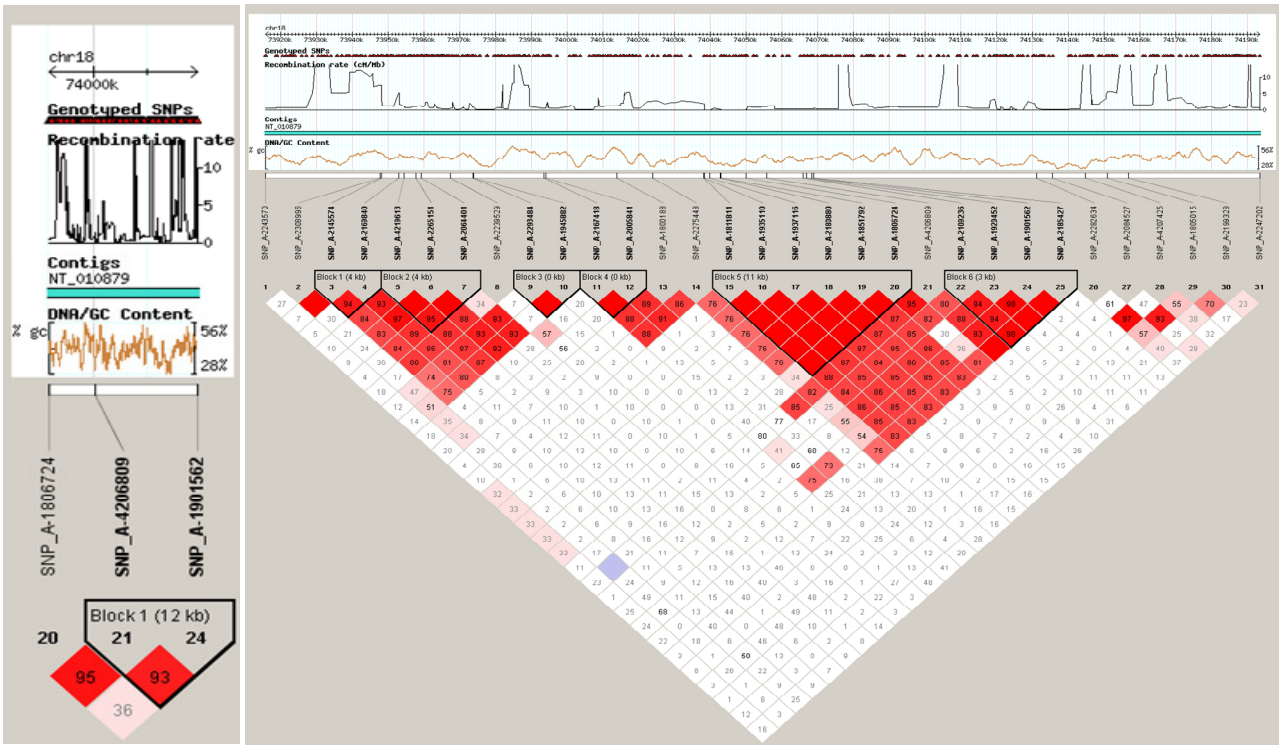


Table 6. 40. *Association between one haplotype which include the most associated marker on chromosome region 18q23 with UAE including age, sex, BMI, SBP, glucose, eGFR by MDRD and number of drugs as co-variables.*

SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	BETA	STAT	P-VALUE
SNP_A-1806724 (rs1039609)	0.408/0.383	0.978	-5.83	0.196	14.4	2.53	0.0121
SNP_A-4206809 (rs1876021)	**0.129/0.104**	**0.996**	**0**	*****	**42.7**	**4.87**	**2.42e-006**
SNP_A-1901562 (rs1039611)	0.196/0.208	0.983	12.5	0.528	21.8	3.08	0.00243

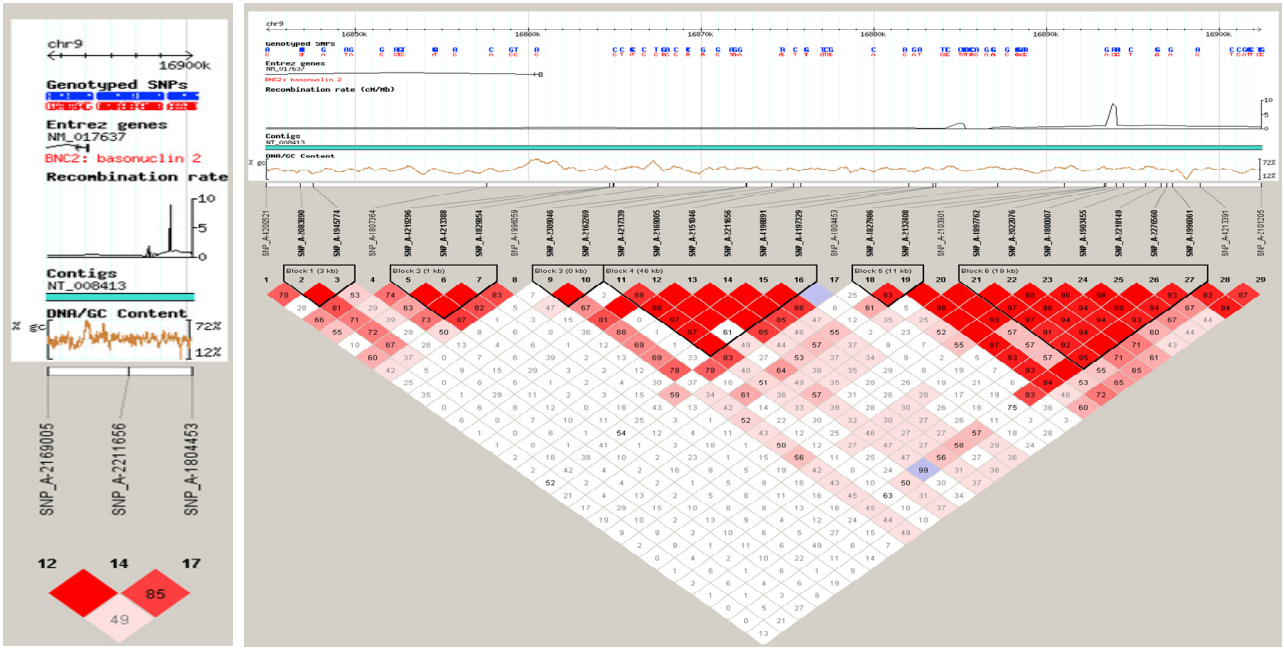
HAPLOTYPE .*.	FREQ	BETA	STAT	P-VALUE
BBB	**0.122**	**42.8**	**4.88**	**2.34e-006**
AAB	0.0703	-12.1	-1.02	0.31
BAA	0.282	-2.67	-0.418	0.677
AAA	0.515	-11	-1.97	0.0499

SNP_A-1806724, **SNP_A-4206809**, SNP_A-1901562

The association with the haplotype which include the significantly associated marker on chromosome 8 remained without changes after including the number of drugs as co-variable.

The haplotype association analysis for the hit on chromosome 9 and the LD of the associated region is shown in table 6.41 and figure 6.36.

Figure 6. 36. *LD of the associated region on chromosome 9.*



As you can see the *SNP_A-2169005* is inside one haploblock in LD with others SNPs, although there are not other SNPs with R-sq >0.8 with it. The marker *SNP_A-2211656* is in high LD with *SNP_A-4198891* which is a Tag-SNP for *SNP_A-4117339* and *SNP_A-2151046*.

Table 6. 41. *Association between one haplotype which include the significantly associated marker on chromosome region 9p22.2 with UAE including age, sex, BMI, SBP, glucose, eGFR by MDRD and treatment as co-variables.*

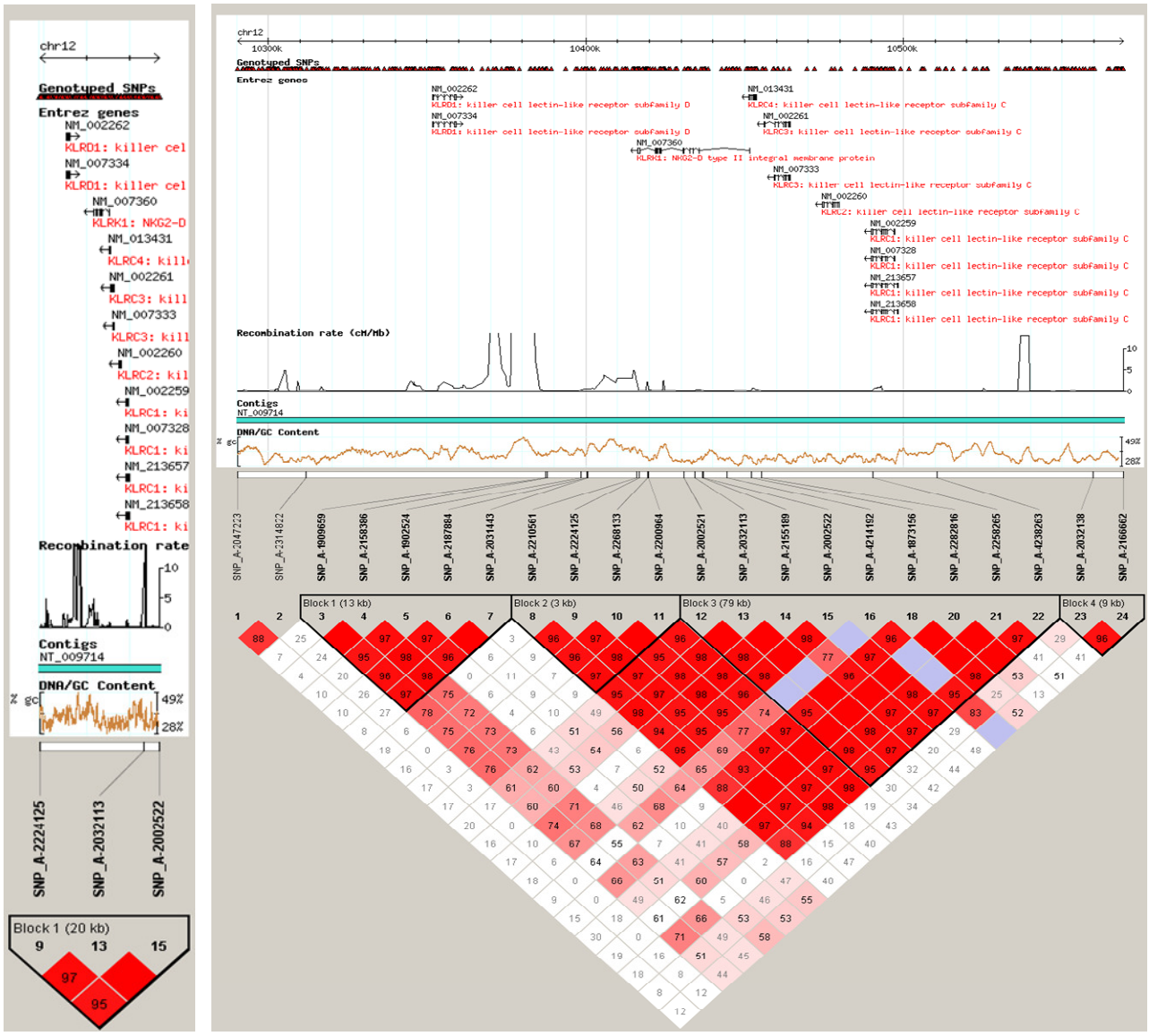
SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	BETA	STAT	P-VALUE
SNP_A-2169005 (rs1339550)	0.117/0.150	0.987	0	*	46.2	5.19	5.61e-007
SNP_A-2211656 (rs3927680)	0.465/0.354	0.991	32.5	0.154	11.6	1.89	0.0608
SNP_A-1804453 (rs10810668)	0.205/0.200	0.991	57.6	0.114	15.1	2.01	0.0462

HAPLOTYPE *..	FREQ	BETA	STAT	P-VALUE
BAB	0.068	54.4	4.63	7.04e-006
AAB	0.122	-10.7	-1.07	0.287
AAA	0.225	-5.66	-0.75	0.454
ABA	0.521	-12.5	-2.01	0.0458

SNP_A-2169005, SNP_A-2211656, SNP_A-1804453

For the associated SNPs on chromosome 12, the haplotype analysis did not increase significantly the strength of the individual association (Table 6.42). The LD of the region on chromosome 12 cytoband p13.2 is shown in figure 6.37.

Figure 6. 37. *LD of the associated region on chromosome 12.*



As we expected for the association of several SNPs in the same region, they are all in high LD between them (R-sq>0.8).

Table 6. 42. *Association between one haplotype which include the associated marker on chromosome region 12p13.2 with UAE including age, sex, BMI, SBP, glucose, eGFR (MDRD) and number of drugs as co-variables.*

SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	BETA	STAT	P-VALUE
SNP_A-2224125 (rs1351113)	0.28/0.305	0.943	-17.7	0.528	16.8	2.52	0.0125
SNP_A-2032113 (rs1382264)	**0.176/0.167**	**0.978**	**0**	*****	**34.2**	**4.28**	**3.01e-005**
SNP_A-2002522 (rs2733840)	0.174/0.167	0.965	2.59	0.985	34.2	4.28	3.01e-005

HAPLOTYPE	FREQ	BETA	STAT	P-VALUE
.*.				
BBA	**0.178**	**35.8**	**4.37**	**2.06e-005**
BAB	0.108	-12	-1.2	0.233
AAB	0.708	-16.5	-2.51	0.0129

SNP_A-2224125, **SNP_A-2032113**, SNP_A-2002522

All of these SNPs on chromosome 12 are in the killer cell lectin-like receptor subfamily K, member 1 (KLRK1) gene: two of them in introns and another in the 3'UTR extreme of the gene. As I said before, this gene is expressed in the natural killer cells and therefore has a role in the immune response system.

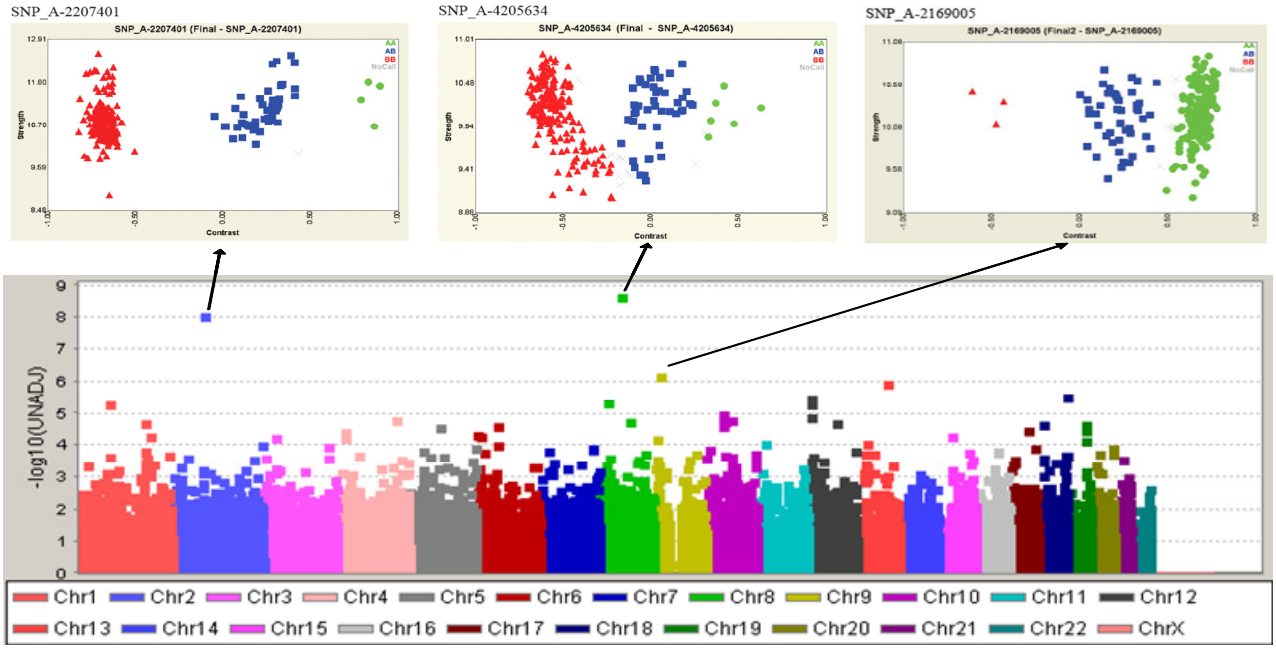
6.3.5.1.2. Models without number of drugs as co-variable

6.3.5.1.2.1. Individual SNPs analysis

The results obtained in the unadjusted analysis were consistent and remained without change after including the following clinical co-variables in the model: age, sex, BMI, SBP, glucose and eGFR by MDRD. The results did not change when considering CC by the Crockoft-Gault formula, DBP instead of SBP or model with less number of co-variables. The first most associated marker was *SNP_A-4205634* (*rs41534844*) on chromosome 8 (p-value unadjusted=2.08×10^{-9}, Bonferroni p-value=0.00035, for the model including age, sex, BMI, SBP, glucose and eGFR by the MDRD formula) and the second, *SNP_A-2207401* (*rs11675985*) on chromosome 2 (p-value unadjusted=8.0×10^{-9}, Bonferroni p-value=0.0013, for the model including age, sex, BMI, SBP, glucose and eGFR by the MDRD formula).

The Manhattan plot for this analysis is shown in figure 6.38.

Figure 6. 38. *Manhattan plot for the association analysis with UAE including age, sex, BMI, SBP, glucose and eGFR (MDRD) as co-variables.*



6.3.5.1.2.2. Haplotype and LD analysis

The association with the previously described haplotypes also remained being even a little bit stronger than the previous one without including clinical co-variables (Tables 6.43 and 6.44).

Table 6. 43. *Association between the haplotype which include the most associated marker on chromosome region 8q12.1 with UAE including age, sex, BMI, SBP, glucose and eGFR (MDRD) as clinical co-variables.*

SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	BETA	STAT	P-VALUE
SNP_A-4194885 (rs985942)	0.495/0.460	0.939	-0.627	0.122	13.4	2.41	0.0167
SNP_A-4205634 (rs41534844)	**0.119/0.100**	**0.952**	**0**	*****	**49.7**	**6.42**	**1.13e-009**
SNP_A-2235942 (rs11992681)	0.321/0.	0.978	155	0.0887	13.5	2.06	0.041

HAPLOTYPE .*.	FREQ	BETA	STAT	P-VALUE
AAB	**0.0847**	**64**	**6.13**	**5.32e-009**
ABB	0.104	-9.85	-0.88	0.38
BBB	0.134	-10	-1.01	0.313
ABA	0.277	-7.01	-1.05	0.294
BBA	0.363	-13.8	-2.2	0.0294

SNP_A-4194885, **SNP_A-4205634**, SNP_A-2235942

Table 6. 44. *Association between the haplotype which include the most associated marker on chromosome region 2p11.2 with UAE including age, sex, BMI, SBP, glucose and eGFR (MDRD) as clinical co-variables.*

SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	BETA	STAT	P-VALUE
SNP_A-2207401 (rs11675985)	**0.1/0.083**	**0.999**	**0**	*****	**54.8**	**6.02**	**9.55e-009**
SNP_A-2208736 (rs1863052)	0.469/0.429	0.999	0.383	0.0988	-1.31	-0.223	0.823
SNP_A-1801207 (rs12714182)	0.443/0.408	0.999	24.7	0.0725	11.2	1.96	0.0512

HAPLOTYPE *..	FREQ	BETA	STAT	P-VALUE
BBA	0.313	-4.29	-0.691	0.49
AAA	**0.0847**	**57.1**	**5.85**	**2.23e-008**
BBB	0.156	4.71	0.586	0.558
BAB	0.385	-17	-2.85	0.00494

SNP_A-2207401, SNP_A-2208736, SNP_A-1801207

6.3.5.2. Association analysis for UAE after excluding patients with macroalbuminuria

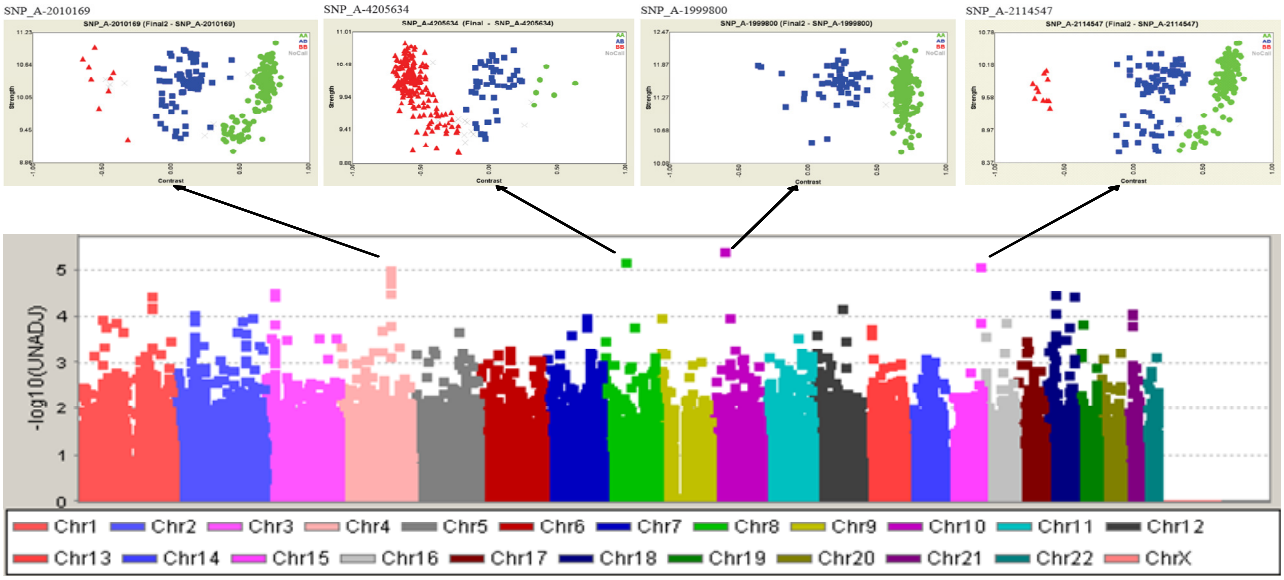
6.3.5.2.1. Models with number of drugs as co-variable

6.3.5.2.1.1. Individual SNPs analysis

For the models which include the number of drugs as co-variable, the most associated SNP was located on chromosome 10, cytoband p11.22, *SNP_A-1999800* (*rs10827234*) although was not significant after correction for multiple comparisons (unadjusted p-value 3.46e-006, Bonferroni p-value 0.5). This marker is located in an intronic region of the neuropilin (NRP1) gene. Interestingly this gene is a membrane bound co-receptor to a tyrosine kinase receptor for both vascular endothelial growth factor (VEGF) and semaphorin and could play a role in angiogenesis and cell survival process.

In this case, the second associated marker was, *SNP_A-4205634*, on chromosome 8, cytoband q12.1 which was significantly associated with UAE without including clinical co-variables, but in this case did not reach the statistical significance (unadjusted p-value 6.02e-006, Bonferroni p-value 1). The Manhattan plot for this analysis is shown in figure 6.39.

Figure 6. 39. *Manhattan plot for the association analysis with UAE excluding subjects with macroalbuminuria (UAE≥300 mg/day) adjusted for age, sex, BMI, SBP, glucose, eGFR (MDRD) and NDRUGS.*



As you can see in the plot another peak was on chromosome region 15q26.2, *SNP_A-2114547* (*rs8029354*), close to the multiple C2 and transmembrane domain-containing protein 2 (MCTP2) gene and there were several SNPs in the same cytoband of chromosome 4 (q28.3) in a region

without known genes. As I commented before these markers are far away from statistical significance.

6.3.5.2.1.2. Haplotype and LD analysis

The haplotype analysis including the associated SNPs did not find any haplotype with equal or higher strength of association than that for the individual marker, except for the region on chromosome 4 in the model which include age, sex, BMI, SBP, glucose, eGFR (MDRD) and NºDrugs as co-variables (Table 6.45).

Table 6. 45. *Haplotype for the markers on chromosome region 4q28.3 and UAE after excluding subjects with macroalbuminuria (UAE≥300mg/day) adjusted for age, sex, BMI, SBP, glucose, eGFR (MDRD) and NºDrugs as co-variables.*

SNP	MAF(our sample/HapMap-CEU)	GENO	KB	RSQ	BETA	STAT	P-VALUE
SNP_A-1799773 (rs4533822)	0.242/0.242	0.991	-34.8	0.931	13.2	3.27	0.0013
SNP_A-2044426 (rs7678041)	0.22/0.242	0.928	0	*	13.8	3.43	0.00076
SNP_A-1979014 (rs13435210)	0.258/0.258	0.973	59.3	0.276	1.86	0.465	0.642

HAPLOTYPE .*.	FREQ	BETA	STAT	P-VALUE
BBA	0.155	3.14	0.645	0.52
AAA	0.0973	-0.522	-0.0803	0.936
BBB	0.0742	33.4	5.03	1.18e-006
AAB	0.661	-11	-2.94	0.00368

SNP_A-1799773, SNP_A-2044426, SNP_A-1979014

6.3.5.2.2. Models without number of drugs as co-variable

6.3.5.2.2.1. Individual SNPs analysis

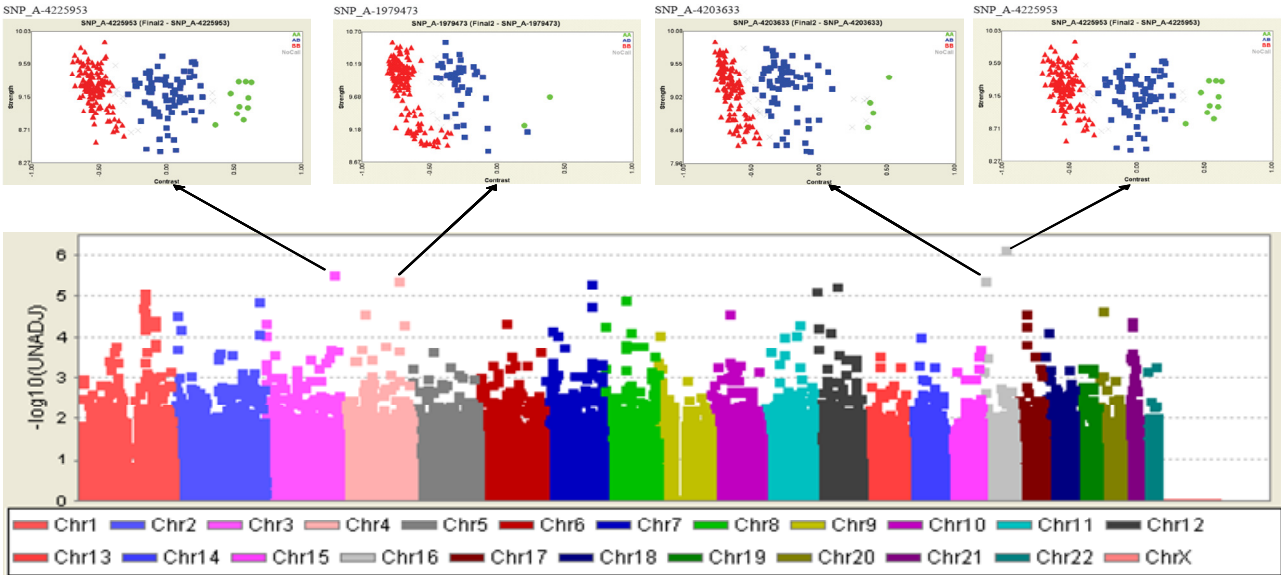
The genomic inflation factor of this model was 1 with a mean chi-square statistic of 0.98. None of the SNPs was significantly associated with UAE after excluding those subjects with UAE≥300 mg/day and when including clinical co-variables in the linear regression model. The only SNP which got a p-value of minus seven was, *SNP_A-4198239* (p-value 6.79e-007, Bonferroni p-value 0.1), in a model which included age, sex, BMI, DBP, glucose and eGFR by the MDRD formula. That polymorphism is located on chromosome 16, cytoband q21, and there is one gene, cadherin

11 precursor (CDH11), within a distance of 150kb upstream. This gene codifies one integral membrane protein which mediates calcium dependent cell-cell adhesion and has been associated with the bone development.

In this model the hit on chromosome 8, *SNP_A-4205634*, was in the eleventh position of the association analysis but it was not significantly associated after correction for multiple testing.

In figure 6.40 you can see the Manhattan plot for this analysis

Figure 6. 40. *Manhattan plot for the association analysis with UAE excluding subjects with macroalbuminuria (UAE≥300mg/day) adjusted for age, sex, BMI, DBP, glucose and eGFR (MDRD).*



6.3.6. Tables of the main results

6.3.6.1. Table 6.46. Main associated SNPs sorted by chromosome

This table summarizes the results for each one of the individual SNPs which have been described in previous pages for the qualitative and quantitative traits, unadjusted and adjusted for clinical co-variables. The statistically significant results are highlighted.

6.3.6.2. Table 6.47. Main associated haplotypes sorted by chromosome

This table summarizes the results for each one of the haplotypes which have been described in previous pages for the qualitative and quantitative traits, unadjusted and adjusted for clinical co-variables. The statistically significant results are highlighted.

Table 6.46. *Main associated SNPs sorted by chromosome.*

		Qualitative traits				Quantitative traits			
Cytoband	Marker	Alb	Malb	Alb adjusted Drugs/No drugs	Malb adjusted Drugs/No drugs	EUA	EUA (No UAE≥300mg /day)	EUA Adjusted Drugs/No drugs	EUA (No UAE≥300mg/day)) Adjusted Drugs/No drugs
2p11.2	**SNP_A-2207401 (rs11675985)**	0.0132	0.0393	0.5808/0.0477	0.9336/0.0246	**6.39e-009**	9.93e-004	7.03e-006/ **8.00e-009**	0.0576/0.0035
2p16.1	SNP_A-1889822 (rs10427222)	0.0021	0.0067	2.0e-004/0.0017	1.63e-004/ 8.58e-004	0.4433	0.023	0.1013/ 0.4319	8.42e-005/0.0381
2q32.2	SNP_A-1844966 (rs6719224)	0.0026	0.0036	1.8e-004 /0.0002	2.48e-004/0.0003	0.0082	0.0025	0.0072/ 0.0073	3.49e-004/0.0016
2q32.2	SNP_A-2024873 (rs2033870)	0.0028	0.0058	1.9e-004 /0.0007	1.90e-004/0.0013	0.0047	0.0019	0.0035/ 0.0044	2.05e-004/0.0019
3q27.1	SNP_A-4225953 (rs4859184)	0.0045	0.0028	0.0221/0.0094	0.0124/0.0041	0.0425	1.79e-006	0.5865/ 0.1045	0.0021/ 2.59e-006
4q13.1	SNP_A-2132603 (rs7658065)	0.0695	0.0902	0.2257/0.0636	0.1397/0.0035	0.0553	3.95e-006	0.5705/ 0.0737	0.0013/ 2.50e-005
4q28.3	SNP_A-2010169 (rs4447914)	0.215	0.2954	0.0033/0.0246	0.0028/0.0231	0.0945	0.0173	0.0137/ 0.0716	8.72e-006/0.0266
4q28.3	SNP_A-1839298 (rs11734157)	0.2333	0.3173	0.0042/0.0274	0.0033/0.0258	0.084	0.0134	0.0136/ 0.0621	1.12e-005/0.0200
4q28.3	SNP_A-2044426 (rs7678041)	0.1142	0.1613	0.0020/0.0101	0.0016/0.0095	0.0212	5.36e-004	0.0138/ 0.0155	1.68e-005/ 7.16e-004
4q32.1	SNP_A-1979473 (rs13116186)	0.555	0.4132	0.1195/0.2562	0.0957/0.1887	0.0586	3.92e-005	0.2608/ 0.0432	0.0026/3.61e-006
5q23.2	SNP_A-2307844 (rs11241841)	5.01e-005	2.64e-005	5.0e-004/3.33e-005	1.25e-004/ 1.92e-005	0.0455	9.43e-004	0.2021/ 0.0756	8.10e-004/0.0023
5q23.2	SNP_A-4211251 (rs13180385)	0.1083	0.1363	2.0e-004/0.0003	1.37e-004/0.0001	0.0766	0.0022	0.0713/ 0.0619	1.83e-004/ 7.44e-004
5q23.2	SNP_A-1983348 (rs327866)	0.013	0.0052	0.0041/0.0004	0.0011/ 9.53e-005	0.4649	0.0045	0.8914/ 0.5013	0.0058/0.0124
7p14.1	SNP_A-2099344 (rs1721936)	7.92e-005	3.00e-004	0.0038 /4.31e-005	0.0025/4.11e-005	0.004	7.80e-005	0.1473/ 0.0089	0.0028/ 7.20e-004
7p14.2	SNP_A-1989536 (rs7778558)	0.0208	0.0141	0.0013/0.0002	0.0007/ 9.39e-005	0.0554	1.78e-005	0.7256/ 0.0444	0.0118 / 8.14e-005
8p11.21	SNP_A-2151676 (rs11787055)	0.0357	0.0071	2.0e-004/0.0031	1.32e-004/0.0017	0.7778	0.2698	0.5322/ 0.5927	0.0384/0.1203
8p21.2	SNP_A-1992707 (rs4872244)	0.0031	0.0075	0.0030/3.219e-005	0.0075/9.62e-005	0.0031	0.0753	0.0054/ 0.0017	0.0228/0.0343
8p21.2	SNP_A-1911590 (rs6557750)	0.00687	0.0222	0.0015/3.83e-005	0.0063/0.0056	6.88e-005	0.0303	7.87e-006/4.16e-006	0.0033/0.0156
8p22	**SNP_A-4215311 (rs6980941)**	**4.29e-008**	1.74e-007	0.3704/0.0644	0.2992/0.0442	0.4967	0.0214	0.7543/ 0.6815	0.3055/0.0541
8q12.1	**SNP_A-4205634(rs41534844)**	0.0004	0.002	0.0415/0.0013	0.0953/0.0071	**1.17e-009**	1.34e-006	1.10e-007/ **2.08e-009**	6.02e-006/ 1.16e-005
9p22.2	**SNP_A-2169005 (rs1339550)**	0.3641	0.7906	0.0867/0.0834	0.3624/0.0590	1.30e-006	0.6316	**1.92e-007**/ 6.20e-007	0.2544/0.3875
10p11.22	SNP_A-1999800 (rs10827234)	0.176	0.1757	0.0035/0.0580	0.0077/0.0084	0.0341	0.0093	0.0018/ 0.0288	3.46e-006/0.0096
10q11.21	SNP_A-4204317 (rs2154401)	2.52e-006	1.27e-006	0.0255/0.0005	0.0078/9.74e-005	0.7217	1.36e-004	0.6268/ 0.8213	0.0070/8.73e-004
11p12	SNP_A-2010350 (rs3552231)	0.0107	0.0034	2.1e-004 /0.0036	1.24e-004/0.0018	0.8166	0.1891	0.5316/ 0.8239	0.0165/0.2655
12p13.2	SNP_A-2260232 (rs17808125)	0.679	0.0574	0.0325/0.0627	0.0315/0.0081	2.97e-004	2.82e-006	0.0059/ 2.76e-004	2.16e-004/ 6.92e-006
12p13.2	SNP_A-2002522 (rs2733840)	8.00e-004	0.0178	0.0019/0.0115	0.0084/0.0067	1.09e-005	0.0031	3.28e-006/ 4.38e-006	5.27e-004/0.0050
12p13.2	SNP_A-2002521 (rs2733845)	0.0024	0.0188	0.0019/0.0168	0.0128/0.0052	1.02e-005	0.0035	4.09e-006/ 2.89e-006	0.0014/0.0047
12p13.2	SNP_A-2032113 (rs1382264)	0.0055	0.0385	0.0019/0.0214	0.0078/0.0103	2.51e-005	0.0052	5.59e-006/ 1.12e-005	7.30e-004/0.0073
12q24.23	SNP_A-1888702 (rs7974380)	2.89e-006	6.12e-006	0.0030 /0.0019	0.0027/0.0016	0.2773	0.0635	0.3187/ 0.2953	0.0270/0.0861
15q26.2	SNP_A-2114547 (rs8029354)	0.0097	0.0101	0.0037/0.0029	0.0044/0.0027	0.0095	7.53e-005	0.0273/ 0.0222	7.63e-006/ 1.68e-004
16p13.2	SNP_A-4203633 (rs17191965)	0.0115	0.0118	5.0e-004/0.0011	0.0006/0.0012	0.0036	1.78e-005	0.0368/ 0.0025	2.34e-004/ 3.76e-006
16q21	SNP_A-4198239 (rs17410049)	0.8562	0.852	0.0281 /0.0107	0.0209/0.0697	0.072	3.85e-005	0.2063/ 0.0506	1.16e-004/ 6.79e-007
16q23.1	SNP_A-2194559 (rs146509)	0.067	0.0816	3.219e-005/0.0115	1.30e-004/0.0056	0.7599	0.4678	0.5382/ 0.8041	0.0212/0.3901
18q23	**SNP_A-4206809 (rs1876021)**	0.0299	0.1433	0.0351/0.1016	0.0951/0.0404	2.74e-006	0.0311	**5.49e-008**/2.59e-006	3.31e-005/0.0187

Chr1 Chr2 Chr3 Chr4 Chr5 Chr6 Chr7 Chr8 Chr9 Chr10 Chr11 Chr12
Chr13 Chr14 Chr15 Chr16 Chr17 Chr18 Chr19 Chr20 Chr21 Chr22 ChrX

Statistically significant results are highlighted.

Table 6.47 *Main associated haplotypes sorted by chromosome.*

		Qualitative traits					Quantitative traits			
Cytoband	Markers	Haplotype	Alb	Malb	Alb Adjusted Drugs/No drugs	Malb Adjusted Drugs/No drugs	EUA	EUA (No UAE≥300mg/day)	EUA Adjusted Drugs/No drugs	EUA (No UAE≥300mg/day) Adjusted Drugs/No drugs
2p11.2	**rs11675985, rs1863052, rs12714182**	**AAA**	0.0172	0.0446	0.0616/ 0.0616	0.28/ 0.28	**1.52e-008**	9.94e-004	**9.55e-009/ 2.23e-008**	0.00121/ 0.00121
2q32.2	rs4667295, rs1550388, rs6719224	BAB	0.00131	0.00191	1.27e-004/ 5.05e-005	2.34e-004/ 9.87e-005	0.00726	0.00211	0.00634/ 0.00634	0.00173/ 0.00173
3q27.1	rs4859184, rs6808204, rs1911979	ABA	0.00573	0.00386	0.00328/ 0.00328	0.00145/ 0.00145	0.0388	6.25e-006	0.0443/ 0.0443	3.15e-006/ 3.15e-006
4q13.1	rs7658005, rs12509018, rs1451170	BBB	0.0627	0.0902	0.0316/ 0.0316	0.0159/ 0.0159	0.0553	3.18e-004	2.34e-006/ 0.0571	4.33e-006/ 4.33-006
4q28.3	rs4533822, rs7678041, rs13435210	BBB	0.236	0.257	0.0236/ 0.0236	0.0232/ 0.0232	0.0238	7.03e-004	0.0252/ 0.0252	1.18e-006/ 7.82e-004
5q23.2	rs11241841, rs2568395, rs326041	ABA	0.00202	0.00115	1.68e-005/ 6.16e-006	2.45e-006/ 8.91e-006	0.0534	0.00131	0.0544/ 0.0544	0.00134/ 0.00134
5q23.2	rs11241841, rs327866, rs7727284	AAA	0.00202	0.00115	1.68e-005/ 1.68e-005	8.91e-006/ 1.96e-006	0.0534	0.00131	0.0544/ 0.0544	0.00134/ 0.00134
7p14.1	**rs1721396, rs1721393, rs1403986**	**BBA**	3.76e-005	2.18e-004	2.38e-005/ 2.8e-005	2.05e-005/ 2.05e-005	0.00432	8.92e-005	0.00521/ 0.00521	1.21e-004/ 1.21e-004
7p14.1	**rs1668349, rs1721396, rs10280461**	**AAA**	3.76e-005	2.18e-004	2.38e-005/ 2.38e-005	2.05e-005/ 1.96e-006	0.00432	8.92e-005	0.00521/ 0.00521	1.21e-004/ 1.21e-004
7p14.2	**rs4723436, rs7778558, rs10233082**	**ABA**	0.0166	0.0102	1.2e-04/ 1.2e-004	4.44e-005/ 0.00809	0.0563	1.32e-005	0.0542/ 0.0542	1.16e-005/ 1.16e-005
8p21.2	rs9314285, rs12551522, rs4872244	BAB	0.352	0.265	0.673/ 0.673	0.737/ 6.74e-004	0.851	0.921	0.814/ 0.814	0.969/ 0.969
8p21.2	rs12551522, rs4872244, rs6557750	AAA	0.352	0.265	0.673/ 0.00148	0.737/ 0.737	0.851	0.921	0.814/ 0.814	0.969/ 0.969
8p22	**rs351562, rs614439, rs2154401**	**BAA**	**1.2e-007**	**7.51e-007**	0.166/ 0.166	0.126/ 0.126	0.503	0.0728	0.523/ 0.523	0.0801/ 0.0801
8q12.1	**rs985942, rs41534844, rs11992681**	**AAB**	0.00194	0.00512	2.01e-004/ 2.01e-004	0.00186/ 0.00186	**8.04e-009**	**3.49e-008**	**1.05e-009/ 5.32e-009**	1.29e-006/ 1.29e-006
9p22.2	rs1339550, rs3927680, rs10810668	BAB	0.363	0.786	0.178/ 0.178	0.816/ 0.816	1.04e-005	0.633	7.04e-006/ 5.61e-007	0.556/ 0.556
10q11.21	rs1492712, rs2639463, rs2154401	BBA	2.77e-005	1.13e-006	2.32e-004/ 2.32e-004	2.86e-005/ 2.86e-005	0.682	0.00183	0.727/ 0.727	0.00232/ 0.00232
11p12	rs7929217, rs355231, rs996262	BAB	0.0131	0.0037	0.0134/ 0.0052	0.00789/ 0.00304	0.817	0.187	0.81/ 0.81	0.184/ 0.184
12p13.2	rs2742115, rs17808125, rs6488270	BBB	0.67	0.568	0.0811/ 0.0811	0.108/ 0.108	2.85e-004	3.75e-004	2.5e-004/ 2.5e-004	2.11e-006/ 2.11e-006
12p13.2	rs1351113, rs1382264, rs2733840	BBA	0.00134	0.018	0.046/ 0.046	0.159/ 0.159	2.27e-005	0.0048	2.06e-005/ 3.01e-005	0.00587/ 0.00587
12q24.23	rs11068715, rs10850931, rs7974380	ABA	6.08e-006	1.87e-005	7.73e-004/ 7.73e-004	6.09e-004/ 6.09e-004	0.269	0.0605	0.253/ 0.253	0.054/ 0.054
18q23	rs1039609, rs1876021, rs1039611	BBB	0.031	0.146	0.133/ 0.133	0.442/ 0.442	2.74e-006	0.0312	2.06e-005/ 2.42e-006	0.0293/ 0.0293

Chr1 Chr2 Chr3 Chr4 Chr5 Chr6 Chr7 Chr8 Chr9 Chr10 Chr11 Chr12
Chr13 Chr14 Chr15 Chr16 Chr17 Chr18 Chr19 Chr20 Chr21 Chr22 ChrX

Statistically significant results are highlighted.

Discussion

7. DISCUSSION

The main results of our study are summarized in tables 6.46 and 6.47.

A few polymorphisms have resulted in an association with the presence of an increment in UAE expressed as a qualitative or as a quantitative trait in our cohort of young hypertensive patients. These SNPs are: *rs6980941* in cytoband p22 of chromosome 8, *rs41534844* in cytoband q12.1 of chromosome 8, *rs11675985* in cytoband p11.2 of chromosome 2, *rs1339550* in cytoband p22.2 of chromosome 9 and *rs1876021* in cytoband q23 of chromosome 18.

There was another group of SNPs which did not reach the statistical significance but they seemed to stand out from the rest of SNPs. It is possible that some of them were false negative because of the lack of statistical power. However they were also considered as potential candidates because they were previously linked with renal damage.

7.1. Method

We have performed a genome wide scan in 302 young hypertensive patients selected from a cohort with a long follow-up to assess the main factors associated with the development of microalbuminuria.

GWAS is a challenging task because there are a great number of potential factors which can affect the final result including: subjects (phenotyping), genotyping and statistical analysis. In the following paragraphs I will discuss some of these issues in detail.

7.1.1. Subjects

A correct definition of phenotype, sample size and homogeneity of the sample are among the main factors which can influence the results in GWAS.

First, the sample size which is difficult to establish and has to be calculated based on factors that we do not know a priori including: a) the genome coverage of the chip which has to include a marker in linkage disequilibrium (LD) with the susceptibility variant; b) the magnitude of the LD value between the susceptibility variant and the marker; c) the MAF for both the marker and the susceptibility allele, *d*) the OR associated to the susceptibility variant and e) the disease frequency. Although we had a small sample size after exclusion of low quality samples, while the study can be made underpowered a priori, the accuracy of the phenotype can minimize this problem. By using only markers with a MAF higher than 0.1 and also analyzing also the urinary albumin excretion as a quantitative trait we tried to improve the power of the study.

Secondly, the ethnical homogeneity of the sample. A different genetic background between cases and controls introduces bias. Statistical analyses have to be done in order to detect subjects with different origins. The genomic control, the multidimensional scaling plot for the IBS pairwise distances and the Eigenstrat analysis are the most commonly used. In our case, all subjects included were of Caucasian origin and belong to the same geographical area. After applying the filters and excluding those samples with very high IBS or those individuals who did not cluster as the rest in the multidimensional scaling plot for the IBS pairwise distance, our genomic control was close to 1.0. This genomic inflation factor did not reveal evidence of any kind of stratification so we can be sure that we do not have bias due to population stratification in our results.

Thirdly, a correct definition of the study phenotype in order to avoid the misclassification bias. This is one of the strengths of our study because our patients were selected from a cohort study in young hypertensive patients with a tight follow-up which significantly reduced misclassification bias. In addition, the UAE was assessed in 24 hour urine sample and on more than one visit. Moreover in those cases in which there were discrepancies between measurements, a third determination was made.

Although we are quite confident that our patients were correctly classified at the time of inclusion we cannot assure that controls will not develop micro or macroalbuminuria or that some of the microalbuminurics patients will not develop macroalbuminuria in the future. This can be especially important for those patients with UAE levels close to the microalbuminuria threshold (30 mg/dl) or to the macroalbuminuria threshold (300 mg/day). This problem can be solved by selecting hypercontrols with no UAE at all and cases not too close to the upper limit. In our case the majority of the controls, 169 (84.1%) had an UAE ≤ 15 mg/day and the majority of the cases, 77 (75.5%), had an UAE ≥30 mg/day and ≤ 161 mg/day. Because we included 8 individuals with macroalbuminuria (UAE≥ 300mg/day) we performed the analysis with and without those subjects and the results were slightly different. We do not know if the susceptibility loci for micro and macroalbuminuria are or not the same or not but the inclusion of the macroalbuminuric patients ensured that some but not all the signals for microalbuminuria reached the statistical significance.

Finally, other factors related with the patient status regarding hypertension and glucose metabolism need to be considered.

One of them is regarding to the blood pressure (BP) levels. Although we only included hypertensive patients with BP levels within the range 135-160 of SBP and 85-100 mmHg of DBP to try to reduce the mechanical influence of BP in the renal damage, the ANOVA test revealed significant differences for 24 hour blood pressure levels and also for glucose being significantly higher in cases than in controls. These factors along with the time of evolution of high BP and with differences in treatment are among the factors that can explain the development of renal damage and might have interfered with the results. The long term of observation of the patients minimizes this problem as well as the fact that the kind of drugs did not influence UAE at long term.

7.1.2. Genotyping

In GWAS, problems affecting the genotyping procedure can have a great impact on the final results. Quality of the DNA samples, management of the samples in the laboratory following the instruction of the manufacturer with a pre-PCR and a post-PCR room to avoid the sample contamination, size and composition of the batches and algorithms to transform the intensity files into the genotypes are all potential sources of problems. Nowadays we usually trust in experimented centres to have the genotyping done. It is important to have a tight contact with the genotyping centre and to obtain from them all the quality parameters to find out the source of possible problems affecting the downstream analysis.

Although microarray is a high throughput technology, our results faced the traditional problems of this technique. A DNA problem was observed with some of the samples, although the amount of DNA and the ratio 260/280 were within the optimal range. The fact that the call rate of some of the

repeated samples were similar, makes us to think that in those samples the DNA integrity was corrupted. We excluded those samples with DNA problem.

Another source of problems is due to the algorithm to transform the intensity files into the genotypes. The accuracy of these algorithms is very high when the call rate of the sample is close to 100% but not for those samples with a call rate far from 100%. In this last case some individuals can be assigned a wrong genotype or a missing genotype. As we selected 90% for individuals and SNPs call rate due to our screening purposes, there is a group of SNPs (especially those under 95% of call rate) with some individuals wrongly assigned to one genotype instead of the right one as seen in some of the cluster plots. Ideally all the SNPs should be reviewed manually, but this is costly and time consuming. The replication of the associated SNP with a different genotyping technology will confirm or negate our results.

7.1.3. Statistical analysis

One of the main problems in GWAS is the possibility of false positive results. This is due to not only to the multiple comparisons but also to problems affecting the genotyping or the phenotyping which can influence all the downstream analysis. It is extremely important to be sure before starting the statistical analysis that the possible differences in genotypes are due to differences in the main phenotype and not to other reasons such as batch effect, population stratification, misclassification, etc. To address with the multiple comparison problem there are several statistical methods, Bonferroni and the false discovery rate (FDR) being the most commonly used. We decided to use the Bonferroni criteria to considerer one SNP as statistically significant although this method is the most stringent one. Due to our low statistical power, especially in the analysis which included covariables, and the possibility of false negative for some of the results close to the significance level we did not discard a group of SNPs which although they did not reach the statistical significance after Bonferroni correction they clearly stood out from the rest.

The most important thing in genetics in order to assess if one result is a false positive or not, is its replication in an independent cohort. Until the replication analysis does confirm these associations we cannot assume that the associated genome areas are involved.

There are not pre-specified thresholds to filter SNPs and individuals in GWAS and they vary according to the particular characteristics of the study. In our case we decided to use 90% for the individual and SNP call rate because of the microarray platform used and the distribution of call rates. As I commented on before we used 0.1 as a threshold for MAF in order to gain statistical power.

There are some points that I would like to remark on regarding to the interpretation of the results. Some of them raise the hypothesis of a false positive result:

1- There were significant differences for the call rate of the main associated marker for albuminuria and microalbuminuria between cases and controls. Then, there were significantly more missing values in controls than in cases although the average call rate for the SNP was above the threshold (0.90).
2- In some of the associated areas there was only one single polymorphism associated. This can be easily explained because there were not other SNP in high LD with the most associated one and also because the Nsp chip does not cover that region extensively.
3- The quality of some of the main hits as it has been shown in the cluster plots is poor, with a great number of missing individuals (call rate 90%) and misclassification.
4- The main association for albuminuria-microalbuminuria in the whole genome disappeared when we included in the model those factors mainly involved in the development of albuminuria. This was not the case for the quantitative trait in which the main hit remained after the inclusion of clinical co-variables.
5- Although we used a high-density chip with more than 250000 markers with a good coverage of the genome according to the provider, not all the genomic common variants are included and neither are the rare variants. It is possible that the coverage of the main associated areas were not good enough. If this is the case, then we can not narrowly define the area of study which makes the search for candidate genes more difficult.
6 -Another point to take into account is that the results for the UAE in the whole genome showed peaks in other areas of the genome. It is known that the loci that can be related to the presence of one disease and those associated with the levels of the quantitative variable (QTL) might not be exactly the same. For instance it can be possible that the variants related with the presence of renal damage and the variants which regulate the UAE were in different chromosomes.

All these facts raise the hypothesis of a possible false positive, but as we commented on before, this study planned to be the first stage of a two stage design study. We have selected the most significantly associated markers in order to be replicated with another genotyping technology in a larger sample.
Nevertheless, there are other points that are in favour of our results:
1- The association analysis using haplotypes instead of individual markers make the results more consistent although we have to consider that in our case, the number of patients for every haplotype is quite small. For this reason we only considered those haplotypes with a minor haplotype frequency of at least 5% in order again to increase the statistical power.
2- Although after the inclusion of clinical co-variables the statistical power was significantly reduced, in the case of the quantitative trait, the main association remained which reinforces the

results. For albuminuria and microalbuminuria it is possible that the lack of statistical power after including clinical co-variables was responsible for the disappearance of the main associated areas.
3- Some of the associated markers are located within possible candidate genes or close to them such as: MSR1, ADAM7, CXCL12, PEBP1, LOX1, Neuropilin 1, BNC2, CDH11, ELMO1, ELMO3, etc. Moreover some of these areas have been previously described in the literature by different authors and by using different strategies.
4- Based on our results, we have been able to select an interesting panel of SNPs for replication in a larger sample.

7.2. Results

The main analysis discovered a region on chromosome 8 that could be related to the presence of albuminuria and microalbuminuria in hypertensive patients. One SNP (*rs6980941*) and one haplotype, in the cytoband p22 of chromosome 8 showed a significant association with the risk of albuminuria and microalbuminuria even after Bonferroni correction or after running the adaptative permutation analysis. We also found another two markers, one on chromosome 10 cytoband q11.21 (*rs2154401*) and another on chromosome 12 cytoband q24.23 (*rs7974380*) which also might be related to albuminuria and microalbuminuria but they were not statistically significant after the correction for multiple comparisons. Three haplotypes containing the main hits were also associated with albuminuria and microalbuminuria. The analysis including covariates did not reveal any marker significantly associated using Bonferroni correction but some of these areas could be important as much as they have been reported in the literature.

Regarding to the association with UAE as a continuous trait two markers reached the statistical significance. One of them, *rs41534844*, was also on chromosome 8 although in this case not in the short but in the long arm of the chromosome (8q12.1) and another, *rs11675985*, on chromosome 2 cytoband p11.2. Also there was an association with two haplotypes containing these SNPs. After excluding those subjects with UAE>300mg/day, the association with the marker on chromosome region 8q12.1 remained especially when considering the haplotype analysis but not with the marker on chromosome 2p11.2.

If we include clinical co-variables in the analysis, the marker in 8q12.1 remained significantly associated even after Bonferroni correction whatever model we considered and also the association with the haplotype. The marker on chromosome 2p11.2 and also the haplotype including this SNP remained significantly associated but only in those models which did not include the number of drugs as co-variable. If we considered the number of drugs as co-variable, another two markers also reached the statistical significance, one on chromosome 18 cytoband q23 (*rs1876021*) and the other on chromosome 9 cytoband p22.2 (*rs1339550*). This last marker was the third most associated with UAE without including co-variables although in this case it was not statistically significance.

The macrophage acetylated LDL receptor I and II (MSR1) lies quite close to the main hit for albuminuria and microalbuminuria in locus 8p22 and it is an interesting candidate gene because it can be involved in the atherosclerosis process. This gene encodes integral membrane glycoproteins that can mediate the endocytosis of the modified LDL which is a crucial step in the development of the atherosclerosis plaque. It is known that the characteristic histological changes of the renal damage associated to hypertension (glomerulosclerosis) are quite similar to those observed in the atherosclerosis process[277, 278]. Glomeruli inflammation has a key role in the development of albuminuria and MSR1 could be involved in this process. Moreover, one study in

rat models of diabetic nephropathy demonstrated a lack of progression of the typical lesions which appeared in DN including UACR in those rats which lacked the MSR1 gene[279]. Besides as it has been described in the introduction those genes related to the lipid metabolism are thought to be related with the damage due to the atherosclerosis and therefore might be associated with the development of micro or macroalbuminuria in HTN[142]. These kinds of genes have also been previously identified by linkage studies as associated to renal disease [140, 142].

In one of those linkage studies for non diabetic ESRD in blacks, the authors also found a linkage peak on chromosome region 8p22 (*D8S1106*) especially in those pedigrees with the largest BMI (LOD score 3.4)[280]. The associated marker in locus 8p22 in that study, however, was far away from our peak. In this study they also found one marker in region 8p21.2 (*D8S1771*) and another in region 2q32.1 (*D2S1391*) associated with ESRD considering the interaction with age at the diagnosis of ESRD. Two of the most associated markers with albuminuria when we co-variate the analysis by age, sex, BMI, SBP, glucose, and eGFR (MDRD) were also in 8p21.2 (*rs4872244*, and *rs6557750*) although they were not statistically significant. Our markers are approximately 500kb of distance for the associated marker in that linkage study[280]. These markers are quite close to the several genes of the ADAM family, including ADAM7, ADAMDEC1 and ADAM28. The association with one haplotype, which includes one SNP inside ADAM7 gene, was stronger than that for the individual markers. These genes are desintegrin and metallopeptidases which belong to the gene family of membrane-anchored and secreted proteins that have proteolytic and/or adhesive properties and therefore can be involved in multiple biological and pathological processes including glomeruloesclerosis[281] and inflammation[282]. In the literature one SNP within the ADAM23 gene in chromosomal region 2q33 was associated with UAE in a family-based association study using the "NHLBI´s Framingham Heart Study" population[154].

When including the number of drugs as a co-variable, two of the most associated markers were on chromosome region 2q32.2, although the p values were again far away from significance and they were too far from the linkage peak of Freedman et al[280].

The second most associated SNP with albuminuria and microalbuminuria is located on chromosome 10 cytoband q11.21 although it did not reach the statistical significance threshold. In this region, the chemokine (C-X-C motif) ligand 12 (CXCL12) is located although it is around 250kb from the main hit in that region. Inflammatory cytokines seem to play a role in the development of renal damage especially in the development of diabetic nephropathy. Rat models of diabetic nephropathy demonstrated over-expression of interferon gamma (INFγ) and tumor necrosis factor alpha (TNF- α) both in the early and late stages of the diabetes nephropathy[283] . Besides in subjects with type I diabetes, pro-inflammatory cytokines such as: interleukin 6 (IL-6), interleukin 8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), interferon-gamma-inducible protein (IP-10),

and macrophage inflammatory protein-1delta (MIP-1δ), measured in urine samples, were associated with the decline in the renal function[284] .

The third most associated marker for albuminuria and microalbuminuria (*rs7974380*) was located on chromosome 12, cytoband q24.23, close to several genes (RFC5, WSB2, PEBP1, TAOK3, KSR2). Between them, the phosphatidylethanolamine binding protein 1 (PEBP1) gene through its role in PKC signalling can have a role in the development of diabetes nephropathy[285]. Protein Kinase C (PK-C) belongs to a multigene family which is involved in different signalling pathways and especially in the response to different growth factors. Several polymorphisms of the PK-C beta gene might ease the development of renal impairment in Japanese diabetics[286]. Besides the overexpression of PKC is a potential target to treat the DN[286-289].

Another interesting result for the association with microalbuminuria including co-variables is the finding of one polymorphism in the cytoband p14.2 of the chromosome 7. In this region the ELMO gene is located which was associated with the risk of DN in a GWAS performed in a Japanese population of type 2 diabetes[245, 246]. This gene through its role in the regulation of extracellular matrix proteins seems to be involved in the development of DN[247]. However our peak is far away from the ELMO gene (about 1500 kb) and as I said before, none of this polymorphism reached the statistical significance when including co-variables in the LR model.

Regarding the association analysis with UAE as a continuous trait, we found another two polymorphisms which reached the statistical significance even when Bonferroni correction was applied and also when important clinical co-variables such as age, sex, BMI, SBP, DBP, glucose or eGFR were included in the linear regression model. The most associated marker was located, as in the case of albuminuria and macroalbuminuria, on chromosome 8 but not in the short but in the long arm (8q12.1) (*rs41534844*, Bonferroni p-value 0.00019). This SNP was also the most associated one with UAE after excluding those patients with an UAE≥300mg/day but in this case it did not achieve the statistical significance after adjusting for multiple comparison (unadjusted p-value=1.34×10^{-6}, Bonferroni p-value=0.227), although the haplotype analysis which includes the allele *B* of this SNP was quite consistent (p-value 3.49×10^{-8}). The minor allele of this SNP significantly increased the levels of UAE (Beta 49.8, CI (34.6-65.1)). The inositol monophosphatase domain containing 1 (IMPAD1) gene is very close to the main associated marker. It is not clear what the exact function of this gene is and it has not been reported to be associated with UAE or nephropathy. Another SNP in that region 8q12.1, *rs10504244*, showed an association with cystatin C but not with UACR within the Framingham Heart Study in an American general population of European descent [154]. Those authors also did not find a suitable candidate gene in that region. The distance between that marker and our marker was around 1200kb.

Another marker significantly associated with UAE was on chromosome 2 cytoband p11.2 and it is in an intronic region of the Receptor Expression-Enhancing Protein 1 (REEP1) gene. Mutations in

this gene have been related to autosomal dominant hereditary spastic paraplegia but as in the other gene it has not been related with renal damage until now.

The third most associated SNP for UAE although it was not significant is on chromosome 9 cytoband p22.2. Interestingly, this SNP, *rs1339550*, reached the statistical significance when we included the Nº of drugs as a clinical co-variable (Bonferroni p-value 0.032). This polymorphism is located in an intronic region of the basonuclin 2 (BNC2) gene. Functional studies demonstrated a different expression profile for the BNC1 gene between smooth muscle cells obtained from human coronary artery or from saphenous veins exposed to an oxidized LDL or platelet-derived growth factor[290]. BCN2 in contrast to BCN1 is widely expressed in different tissues including the kidney. It is expressed in the nuclei so it is thought to influence the mRNA. We do not know if the BCN2 might also be involved in the atherosclerosis process and in the renal damage associated to HTN.

Polymorphisms within a near region on chromosome 9 (9p21.3) have been found to be associated with coronary heart disease in GWAS and these results have been widely replicated[88, 291, 292]. Among the genes that could be responsible for this association are the cyclin-dependent kinase inhibitor 2A and 2B (CDKN2A and CDKN2B). These genes are thought to be implicated in cell proliferation and apoptosis which usually occurs in the atherogenesis process. Besides CDKN2A and CDKN2B, in 9p21.3, it is the family of the type I interferon genes which include IFNAs, IFNB1 and IFNW1. These groups of cytokines have pro-inflammatory, antiviral and immunoregulatory properties and therefore are widely used for the treatment of several chronic inflammatory diseases. They have also been related with several autoimmune diseases in animal models including systemic lupus erythemathosus (SLE) and type I diabetes and related traits but not all the studies support that association[293-296]. Although the relationship between type I interferons and renal damage have not been described we can speculate that these group of cytokines might also be involved in the pathogenesis of microalbuminuria not only in diabetes but also in hypertension as a result of the immune response to the endothelial injury in the renal glomeruli. This region 9p21.2 reached the maximum LOD score for hypertensive ESRD in the linkage study performed in Black American families as reported by Freedman et al[280] (*D9S1121*, LOD score 2.03).

Another interesting gene in the short arm of chromosome 9 (9p24.1) is the interleukin 33 (IL-33) gene. This gene was first called “Nuclear factor from High Endothelial Venules”[297] . The IL-33 seems to play a role in trauma, infections and chronic inflammatory diseases by means of the interleukin 1 (IL-1) receptor related protein ST2 and might be involved in the immune response[297]. Within its functions are the drive of the production of pro-inflammatory cytokines especially in the mast cells and lymphocytes T2 and the chemotaxis of Th2 cell[298-300] . The endothelial cells of chronically inflamed tissues seem to be a major source of IL-33 in several diseases such as rheumatoid arthritis and Crohn’s disease [148]. Besides rodents studies have shown that IL-33 is

expressed by cardiac fibroblast after mechanical stress and by endothelial cells during atherosclerosis [301, 302].

Moussion et al[303] demonstrated that IL-33 has a constitutive and widespread expression in the nucleus of endothelium cells along the vascular tree of normal tissues including the peritubular capillaries of the kidney. Surprisingly these authors were not able to detect IL-33 in the kidney glomeruli[303] . The fact that IL-33 could be detected in fibroblast with myofibroblast characteristics suggests that this protein might also be involved in tissue fibrosis and these authors suggest that IL-33 might be particularly important in the injury during cardiovascular diseases[303] . All of these studies indicate that the IL-33 may act as an endogen alarming signal for the immune system after epithelial or endothelial damage.

All of these studies indicate that genes in the short arm of chromosome 9 might be important in the atherosclerosis process and maybe with the vascular glomerular damage induced by hypertension. In those chromosomal regions there are large areas of high LD so we cannot be sure if the associated SNP is the risk variant or a marker in LD with it.

After excluding subjects with UAE≥300 mg/day, the main associated SNP for UAE was the same on chromosome region 8q12.1 (*rs41534844*). In this case the second most associated for UAE was not on chromosome 2 but in an intronic region of the MCF2L2 gene on chromosome 3 cytoband q27.1. It is noteworthy that other authors also found three SNPs in this gene (*rs684846*, *rs35069869* and *rs35368790*) associated with nephropathy in type 2 diabetic subjects[304]. They did not find an association for known candidate genes in that region such as ADIPOQ and IGF2BP2 genes[304]. This region in the long arm on chromosome 3 has been previously detected by linkage studies[84, 151, 228]. Vionnet et al[230] searching for the possible candidate gene in this region in European population of type 1 diabetes found a strong association with a SNP in the promoter region of the ADIPOQ gene.

Another interesting region for the association analysis with UAE after excluding patients with UAE≥300mg/day was 12p13.2. Our SNP is inside the C12orf59 gene but it is quite close to the oxidized LDL receptor 1 (ORL) gene. As I said genes involved in lipid metabolism might be related with hypertensive glomerulosclerosis. Studies in rats demonstrated an up-regulation of the oxidized LDL receptor in the kidney of Dahl salt-sensitive rats which exhibit glomeruloesclerotic changes [305]. This up-regulation runs in parallel with the glomeruloesclerotic changes and with the decline in renal function in these animals[305]. Other studies in rats support the role of the ORL gene, also called LOX1, in the development of DN[306]. Moreover the blockage of this receptor may reverse the pathological glomeruloesclerotic changes[307]. Moreover polymorphisms of this gene were tried to be associated with type 2 DN but the selected SNPs in this gene were not among the most associated ones[308]. Taking into account these facts these kinds of genes which codify receptors for oxidized LDL such as MSR1 and ORL may be related with hypertensive glomerulosclerosis process.

The fourth marker most associated with UAE after excluding macroalbuminurics was in an intronic region of the EPHA5 gene on chromosome 4q13.1. A linkage peak in this region was previously associated with hypertensive ESRD in Black Americans (LOD score 3.44 for the case of late onset hypertension)[280].

When we included co-variables in the analysis for UAE, another marker (*rs1876021*) on chromosome 18 cytoband q23 was also significantly associated if we included the Nº of drugs as a covariable (Bonferroni p-value=0.0093). There are no genes in that specific region, however, our marker is located between the two flanking markers for a linkage peak associated with eGFR (LOD score 2.10 in Mexican-Americans) within the Family Investigation of Nephropathy in Diabetes (FIND) study[243], which is a multicenter study aim to discover susceptibility variants for DN in different ethnics of type 2 diabetics. Previously, other authors had found in that region 18q22.2-23 a very significant linkage peak for DN according to the Lander and Kruglyak scale[141] . This study performed by Vardali et al[223] in 18 large Turkish families of type 2 diabetes found a LOD score of 6.1 on chromosome 18q22.3-23 between the markers *D18S469* and *D18S58*. Moreover this peak was also replicated in type 2 diabetes Pima indian[84, 224]. The CNDP1 gene which codifies the enzyme carnosinase is thought to be responsible for this linkage peak[225, 226]. This enzyme degrades the dipeptide carnosine which can act as an ACE inhibitor[258, 259], natural reactive oxygen species scavenger[260] and advanced glycation end products (AGEs) breaker, therefore it can protect the kidney in diabetic subjects. Although it is a plausible candidate gene at least in type 2 diabetes, a recently published paper in type 1 diabetes failed to find an association between this gene and nephropathy[227]. Our marker is around 4000 kb of distance from that gene so we can not assume that it is the same peak. Anyway in the light of the previous and our results this region (18q22.3-23) deserves special attention.

In the association analysis for UAE adjusted for age, sex, BMI, DBP, glucose and eGFR (MDRD), the most associated marker was on chromosome 16 cytoband q21 although again it was not statistically significant (unadjusted p-value 6.79×10^{-7}**,** Bonferroni p-value 0.1). Near this marker the Cadherin-11 Precursor (CDH11) gene is located. This gene has been especially related with the bone metabolism but because its role in cellular adhesion and calcium ion binding may be implicated in other processes. One gene expression study demonstrated a down-regulation of this gene in the heart of a streptozotocin-induced diabetic rat [309]. This gene is moderately expressed in the glomeruli and vascular smooth muscle cells[310] (http://www.proteinatlas.org/) and it may be involved in atherosclerosis by means of extracellular matrix proliferation. In this region other members of the Cadherin family (CDH8 and CDH5) are also located.

The linkage study of Freedman et al[280] for hypertensive ESRD in Black American families also found a peak on chromosome region 16q21 when considering the interaction with age at diagnosis of hypertension (*D16S1385* (P =0.0084). In the long arm of chromosome 16 there are some

identified candidate genes for DN such as Cholesterol Ester Transfer protein Precursor (CETP) gene and Solute carrier family 12 member 3 (SLC12A3) gene and also there is a gene of the ELMO family (ELMO3).

Another marker on chromosome 10 (10p11.22), *rs10827234*, was the most associated one with UAE after excluding patients with UAE≥300mg/day and after adjusting for different clinical co-variables including the number of drugs. This SNP is in an intronic region of the neuropilin (NRP1) gene which, as I said in the results, is a membrane co-receptor to a tyrosine kinase receptor for both vascular endothelial growth factor (VEGF) and semaphorine. For this reason it might be involved in angiogenesis and cell survival process. A recent study reported a decrease in the NRP1 expression in cell cultures of mouse podocytes exposed to advanced glycation end (AGE) products[311]. These authors also showed that the expression of this gene in the glomeruli is decreased in diabetic db/db mice as compared to their non diabetic littermates[311]. This also occurs in biopsies of DN as compared to the kidney samples of transplant donors[311]. The down-regulation of the NRP1 gene inhibits the migration of podocytes in the same way as the AGE products does. This can ease the adhesion of the glomerular basement membrane to the Bowman's capsule and lead to the development of focal glomerulosclerosis[311]. Another study demonstrated that NRP1 gene can have a key role in the endothelial cell apoptosis process, which usually occurs in hypertension and atherosclerosis[312]. Iyengar et al[313] in one linkage study performed in sibpairs of Caucasian origin with type 2 diabetes found a linkage between one marker in an intronic region of the NRP1 (*D10S1654*) and DN. Later on seven SNPs in this gene were evaluated by TDT by other researchers within a study of 115 candidate genes for DN[237]. Two of them, *rs869636* and *rs2804495*, were nominally significant (p-value =0.047 and 0.027, respectively)[237]. Our SNP, *rs10827234*, is close to the *rs2804495* in intron 2 (only one SNP between them) and it is in LD with it (R-sq = 0.312, D' = 1.000). Besides these association studies, neuropilin antagonist are claimed to be useful for the treatment of hypertensive nephrosclerosis (http://www.faqs.org/patents/app/20080213268).

Other chromosome regions associated with UAE after excluding macroalbuminurics adjusted for age, sex, BMI, SBP, glucose, and eGFR (MDRD), were locus 15q26.2 close to the multiple C2 domains, transmembrane 2 (MCTP2) gene and locus 4q28.3. As I said, these regions were far from significance. MCTP2 gene has not been reported to be associated with renal damage, although it is expressed in the kidney and it is involved in the calcium-mediated intracellular signalling. Within the FIND study[233], there was a suggestive linkage for ACR in African-American families in 15q26.3 (flanking markers *D15S657-D15S642*). The physical distance between that linkage peak and our marker is about 1650 kb. In that region 15q26.3 is the Insulin Growth Factor 1 receptor (IGF1R) gene which is a strong candidate gene for DN[237]. In the 4q28.3 region, there were no genes close to the associated markers. The closest genes belong to the protocadherin

family (PCDH10 and PCDH18) which seem to play a role in the cell-cell connections in the brain. This region has not been associated with renal damage, but one marker (*D4S2286*) showed an association with hypertension in one linkage study performed in Anglo-Celtic Australian sibpairs [314]. This marker is around 4000 kb of our associated markers in that region.

As I described extensively in the introduction, candidate gene, linkage and GWAS have identified different regions of the genome which may be influencing the appearance of albuminuria and microalbuminuria but only a few loci were replicated. These studies reinforce the hypothesis that the organ damage induced by hypertension is a complex process in which several loci plus the contribution of environmental factors are possibly involved.

As far as I know this is one of the first studies of this kind made in hypertensive subjects with or without established renal damage based on UAE levels. Our analysis revealed several areas of the genome which could be related to the renal damage associated to this condition or which might be involved in the regulation of the UAE. As we do not have the replication of these results we can not rule out if the genomic signals are true or in fact they are false positives. All the limitations described above must be considered when interpreting the results.

The genome wide scans have successfully unravelled the genetic susceptibility of certain complex diseases but in others such as essential hypertension, they tend to fail due to several reasons: lack of power to detect variants with very low associated risk, difficulties regarding to the phenotype, lack of complete coverage of all the genome variants, etc. Probably we are faced with the same difficulties when studying the target organ damage associated with hypertension.

The development of new genotyping chips covering more than one million of single nucleotide polymorphisms (SNPs) and copy number variation (CNV) altogether with a more accurate phenotyping in large scale studies, may be the way to discover the genetic bases of essential hypertension and related organ damage.

7.3. Conclusions

1- Several chromosome regions were significantly associated with UAE expressed as a qualitative or quantitative trait.

2- One polymorphism and one haplotype in locus 8p22 were significantly associated with microalbuminuria and albuminuria. The associated markers lie close to or inside to the macrophage acetylated LDL receptor I and II (MSR1) gene which is a potential candidate for microalbuminuria and albuminuria. Polymorphisms and haplotypes were also significantly associated with UAE as a quantitative trait in the following loci: 8q12.1, 2p11.2, 9p22.2 and 18q23. The potential possible candidate genes for UAE within those chromosome regions are inositol monophosphatase domain containing 1 (IMPAD1), Receptor Expression-Enhancing Protein 1 (REEP1), basonuclin 2 (BNC2), and Carnosine dipeptidase 1 (CNDP1).

3- Lesser strength associated loci for microalbuminuria and albuminuria were found in: 10q11.21, 12q24.23, 2q32.2, 11p12, 16q23.1, 8p21.2, 5q23.2, 7p14.1, 8p11.21, 2p16.1 and 7p14.2. Potential candidate genes in these regions for micro and albuminuria are: chemokine (C-X-C motif) ligand 12 (CXCL12), disintegrin and metalloproteinase domain-containing protein 7 precursor (ADAM7), disintegrin and metalloproteinase domain-like protein decysin 1 (ADAMDC1) disintegrin and metalloproteinase domain-containing protein 28 precursor, phosphatidylethanolamine-binding protein 1 (PEBP1), engulfment and cell motility protein 1 (ELMO1) and engulfment and cell motility protein 3 (ELMO3). For UAE these loci were: 3q27.1, 12p13.2, 4q13.1, 10p11.22, 15q26.2, 4q28.3, 16q21, 4q32.1 and 16p13.2. Potential candidate genes within these regions for UAE were: MCF2-transforming sequence-like protein 2 (MCF2L2), oxidized low-density lipoprotein receptor 1 (LOX1), ephrin type-A receptor 5 precursor (EPHA5), cadherin-11 precursor (CDH11), neuropilin-1 precursor (NRP1), multiple C2 and transmembrane domain-containing protein 2 (MCTP2), protocadherin-10 precursor (PCDH10) and protocadherin-18 precursor (PCDH18).

4- These genes are mainly involved in lipid metabolism, inflammation or intracellular signalling which are mechanisms than can lead to an increase in UAE in HTN.

5- GWAS strategy has enabled the replication of some of the previously associated areas with renal damage using the same or different strategies such as linkage studies or candidate gene study and also to identify new potential susceptibility loci.

6- A panel of SNPs potentially associated with microalbuminuria and UAE according to our data and those reported in the literature has been designed for the replication study.

7.4. Future prospects

Those SNPs which have shown association in the genome wide scan, are now being genotyped with another high-throughput technology (SNPlex- Oligonucleotid ligation assay) in a larger sample of 800 young hypertensive patients and in a general population of 1500 subjects. We should be able to observe if the same loci which were associated, still remain associated and the magnitude of the association.

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