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Doctoral Thesis

Novel genetic risk factors associated with three multifactorial traits and their clinical characteristics in Latvian population

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Annotation

Potentially sharing a similar genetic architecture, coronary artery disease (CAD), diabetes mellitus and pituitary adenomas (PA) are multifactorial diseases contributing significantly to increased mortality and morbidity throughout the world. Decades of studies have discovered numerous genetic susceptibility factors for these diseases and high inter-population variability. The aim of this doctoral thesis was to investigate the roles and associated complications of adenosine A3 receptor (*ADORA3*) and glyoxalase 1 (*GLO1*) polymorphisms in the susceptibility of CAD and diabetes, respectively, as well as to examine the genetic basis of pituitary adenoma (PA) in Latvian population.

The CAD study included in the thesis found a protective missense polymorphism rs35511654 in *ADORA3*, which possibly modulates the damage caused by ischemia, or offers protection by reducing inflammation caused by arterial plaques. The second study uncovered the role of three *GLO1* polymorphisms in the activity of Glo1. Both the rs1130534 and rs1049346 polymorphisms were associated with a decreased activity or concentration of the Glo1 enzyme in the blood, but they were not associated with the presence of complications due to diabetes. Genetic studies on PA found that the *SSTR*5 polymorphisms rs34037914 and rs642249 are associated with the presence of somatotropinomas, earlier onset of disease, and worse clinical outcomes in carriers. The rare *MEN*1 polymorphism rs2959656 showed a strong association with clinically significant PA, regardless of the type of adenoma present. Two DRD2 polymorphisms (rs7131056 and rs4938025) are associated with presence of extrasellar growth of tumor in hormones secreting PA. This suggests a deeper involvement of these variants in PA pathogenesis and possibly shows opportunities for drug therapy modulation which currently targets DRD2. The novel findings of these PA studies are particularly interesting since the genetic aspects of the common forms of PA are still under-investigated in European populations. The information from these four published studies provides additional insight into the genetic mechanisms contributing towards CAD, diabetes complications, and PA in Latvian population and could become a part of improved diagnostic systems and targeted medicine.

Anotācija

Koronārā sirds slimība (KSS), cukura diabēts un hipofīzes adenoma (HA) ir multifaktoriālas slimības ar līdzīgu ģenētisko arhitektūru un aizvien pieaugošu saslimstību un mirstību visa pasaulē. Pētījumi, kas ilguši gadu desmitus, atklājuši lielu skaitu šo slimību ģenētiskos riska faktorus un daudz atšķirību starp dažādām populācijām. Šī doktora darba mērķis ir izpētīt adenozīna A3 receptora (*ADORA3*) polimorfismu lomu KSS riska paaugstināšanā, glioksilāzes 1 (*GLO1*) polimorfismu ietekmi uz diabētu un tā komplikācijām un HA ģenētiskos cēloņus, visos pētījumos fokusējoties uz Latvijas iedzīvotāju populāciju.

Viens no pētījumiem, kas veikts doktora darba ietvaros ir atklājis pret KSS aizsargājošu nesinonīmu polimorfismu rs35511654 *ADORA3*, kas, iespējams, modulē išēmijas izraisīto bojājumu pakāpi vai arī novērš KSS, samazinot iekaisuma reakciju arterialajās pangās. Otrs pētījums noskaidroja trīs *GLO1* polimorfismu lomu Glo1 aktivitātē, konstatējot, ka rs1130534 un rs1049346 ir asociēti ar samazinātu enzīma aktivitāti vai tā daudzumu asinīs, bet ne tieši ar diabēta komplikāciju klātbūtni. HA pētījumi noskaidroja, ka *SSTR5* polimorfismi rs34037914 un rs642249 ir asociēti ar augšanas hormonu (GH) sekretējošas HA klātbūtni, agrāku slimības iestāšanos un smagāku klīnisko slimības norisi riska variantu nesējiem. Plašākā pētījumā arī rets nesinonīms *MEN1* polimorfisms rs2959656 uzrādīja būtisku asociāciju ar klīniski nozīmīgu HA.

Tika atklāts, ka divi *DRD2* polimorfismi (rs7131056 un rs4938025) ir asociēti ar HA, kam ir ekstrasellāra augšana hormonus sekretējošās HA, liecinot par *DRD2* plašāku iesaistību HA potoģenēzē un parādot iespēju medikamentu terapijas, kura mērķēta uz *DRD2* modulācijai.

Šie četri publicētie pētījumi papildina informāciju par KSS, diabēta komplikāciju un HA ģenētiskajiem mehānismiem Latvijas populācijā un šī informācija var dot pienesumu uzlabotā diagnosticēšanā un personalizētās medicīnas attīstībā.

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Abbreviations

A1R, A2AR, A2BR, A3R, AR – adenosine A1, A2A, A2B, A3 receptor, adenosine receptor
ACTH – adrenocorticotropic hormone
ADHs – aldehyde dehydrogenases
AGEs – advanced glycation end products
AKRs – aldoketo reductases
AP-2α – activating enhancer-binding protein 2α
ATP – adenosine triphosphate
AUC – area under curve
BMI – body mass index
CAD – coronary artery disease
Chr – chromosome
CNC – Carney complex
CREB – cAMP response element-binding protein
CST – cortystatin
CVD – cardiovascular disease
DNA – deoxyribonucleic acid
dNTP – deoxynucleotide
ddNTP – dideoxynucleotide
E2F4 – early gene 2 factor isoform 4
EGF – epithelial growth factor
FHS – Framingham Heart Study
FIPA – familial isolated pituitary adenoma
GH – growth hormone
GSH – glutathione
GWAS – genome wide association studies
ICD-10 – International Classification of Diseases
IGF-I – insulin-like growth factor 1
Indiv – individuals
INFγ – interferon gamma
IRE – insulin-response element
kb – kilobases
LDL – low-density lipoprotein
LGDB – Latvian Genome Database
MAF –minor allele frequency
MALDI-TOF –matrix-assisted laser desorption/ionization time of flight
MEN – multiple endocrine neoplasia
MG – methylglyoxal
MI – myocardial infarction
mmol/L – millimoles per liter
mPTP – mitochondrial permeability transition pore
MRE – metal-response element
MRI – magnetic resonance imaging
mRNA – messenger ribonucleic acid
N – number
NFPA – non-functional pituitary adenoma
Nrf2 – erythroid 2-related factor 2
OR – odds ratio

PA – pituitary adenoma
PCR – polymerase chain reaction
PIT1 – pituitary-specific positive transcription factor 1
PKA – protein kinase A
PRL – prolactin
rs#### – reference SNP ####
SAP – shrimp alkaline phosphatase
SNP – single nucleotide polymorphism
SST – somatostatin
T2D, T1D – type 2 diabetes, type 1 diabetes
TSH – thyroid-stimulating hormone
X-LAG – Xq26.3 duplication acrogigantism

Genes and proteins

AIP - aryl hydrocarbon receptor interacting protein
AKT - Protein kinase B
ALOX5AP - Arachidonate 5-Lipoxygenase Activating Protein
ANGPTL4 - Angiopoietin Like 4
ANRIL - CDKN2B Antisense RNA 1
APOE, *APOA5*, *APOC3* - Apolipoprotein E, Apolipoprotein A-V, Apolipoprotein C-III
ARHGEF6 - Rac/Cdc42 Guanine Nucleotide Exchange Factor 6
CD40LG - CD40 Ligand
CDH23- cadherin-related 23
CDKN2B, *CDKN2A* - Cyclin Dependent Kinase Inhibitor 2B, 2A
CREB - cAMP response element-binding protein
DRD2 – Dopamine Receptor D2
EBF1 - Early B-Cell Factor 1
ExoI – Exonuclease I E. coli
G i/o – G protein with inhibitory alpha subunit
GLO1 - Glyoxalase I
GNAS - (guanine nucleotide binding protein, alpha stimulating) complex locus
GPR101 - G Protein-Coupled Receptor 101
GRB10 - Growth Factor Receptor Bound Protein 10
Gs/olf – G protein with activating of adenylate cyclase alpha subunit/in olfactory receptors
GTP-ase - guanosine triphosphate hydrolyze
HAGH - Hydroxyacylglutathione Hydrolase
HMG-CoA - 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, HMGCR
IARS - Isoleucyl-TRNA Synthetase
KIF5A - Kinesin Family Member 5A
LDLR - Low Density Lipoprotein Receptor
LOC400684 - Uncharacterized LOC400684
LPL - Lipoprotein lipase
LTA - Lymphotoxin Alpha
LTA4H - Leukotriene A4 Hydrolase
MEF2 - Myocyte Enhancer Factor 2
MEN1 - Menin 1
MGMT - O-6-Methylguanine-DNA Methyltransferase
MKI67 – Antigen KI-67

MTAP - Methylthioadenosine Phosphorylase
mTOR – FK506-binding protein 12-rapamycin-associated protein 1
NR3C1 - Nuclear Receptor Subfamily 3 Group C Member 1
PCSK9 - Proprotein Convertase Subtilisin/Kexin Type 9
PI3K Phosphatidylinositol-4,5-bisphosphate 3-kinase
PI3KCA - Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PIT1 - Pituitary-specific positive transcription factor 1
PKA – Protein Kinase A
PRKAR1A - Protein Kinase CAMP-Dependent Type I Regulatory Subunit Alpha
RBMX - RNA Binding Motif Protein, X-Linked
SP100 - SP100 Nuclear Antigen
SST5TDM5, SST5TDM4 – Somatostatin Receptor 55 consisting of five or four transmembrane domains
SSTR1, *SSTR2*, *SSTR3*, *SSTR4* and *SSTR5* - Somatostatin Receptor 1; 2; 3; 4 and 5
THBS1, *THBS2*, *THBS4* - Thrombospondin 1, 2, 4
TNFRSF13C - TNF Receptor Superfamily Member 13C
TRIP12 - Thyroid Hormone Receptor Interactor 12
USP8 - Ubiquitin Specific Peptidase 8

Introduction

Coronary artery disease (CAD), diabetes mellitus and pituitary adenoma (PA) are multifactorial diseases of similar genetic architecture that are contributing to increased mortality and morbidity rates throughout the world. They are caused by an interaction between numerous environmental and genetic risk factors. Most of the associated environmental risk factors were discovered and intensely studied in the second half of the 20th century. With the advancement of technology, multiple studies have searched for genetic susceptibility factors for these diseases. This has led to tens to hundreds of genes being identified and implicated in CAD, diabetes and PA. Their intermediate risk factors, complications and phenotypic modifications have also been described. Lately, this genetic research has started to impact decision-making in clinical settings since more and more evidence points towards the value of genetic information in improving prognoses, performing risk assessments and even informing treatment strategies or drug doses. However, not all genetic risk factors are known, while even less is known about their interaction with other genetic and environmental risk factors. Since huge inter-population variation exists, a large area for research studies is provided. It is also clear that less attention has been paid to the investigation of the genetics of numerous clinical phenotypes and the outcomes associated with each of the large multifactorial disease groups. Similarly, despite the advancements in GWAS technology, the number of genetic studies conducted on rare multifactorial diseases like PA is limited. In order to increase the general knowledge of genetic risk factors underlying these conditions and by paying special attention to the specific clinical characteristics and sub-phenotypes, the aim of the research was to study the genetic causes and aspects of multifactorial diseases (CAD, diabetes complications and PA) in Latvian population. To achieve the proposed aim of the study, the following tasks were established:

1. To investigate the role of *ADORA3* variations in CAD susceptibility in Latvian population;
2. To study *GLO1* polymorphisms and assess the associated functional Glo1 activity in connection with common type 1 and type 2 diabetes complications;
3. To identify *SSTR5* mutations in pituitary adenoma patients and to study their impact on the clinical characteristics of somatotropinomas;

4. To scan the tag-SNPs of seven candidate genes (*SSTR5, SSTR2, DRD2, GNAS, MEN1, AIP, PRKAR1A*) in search of common mutations causing pituitary adenomas and to understand how they influence phenotypic characteristics in Latvian population.

1. Literature review

1.1 Multifactorial diseases

Most human diseases and disorders result from a complex interplay between multiple genetic and environmental factors (Lander and Schork 1994). These conditions are commonly called multifactorial diseases or disorders. Multifactorial diseases occur when combination of genetic and environmental factors (often not completely known) interact, but these interactions are not yet identified or not known in details. Due to these unknown facts multifactorial diseases are difficult to study, prevent and treat. Uncovering genetic factors underlying mechanisms of multifactorial diseases can lead to better diagnosis, risk prediction, management of healthcare funds, discovery of new drug targets, and understanding of disease prevention as well as improved general and precision treatment of the disease (Vieira 2014).

In multifactorial diseases there is no clear gradation of phenotype. Number of characteristics of multifactorial diseases distinguishes them from Mendelian or sex-dependent disorders (Lobo 2008). The multifactorial disease can be sporadic, with affected offspring born to unaffected parents and when there is aggregation of disease cases in the family, no Mendelian pattern of inheritance can be observed (Mossey 2002). The risk of the disease is modified by environmental factors such as pollution, lifestyle, viral infections and others (Ralston 2008). The disease is more frequent in one sex than in other but is not sex dependent (Lobo 2008). The percentage of twin pairs in which both twins are affected by disease does not follow Mendelian proportions in both monozygotic and dizygotic twins (Mossey 2002). And the disease is more frequent in a specific population (Clark and Emerole 1995).

A classic example of this is coronary artery disease (CAD) with many factors increasing the risk like diabetes, elevated blood pressure and increased LDL cholesterol concentration. CAD does not show Mendelian inheritance although having family member with CAD is one of risk factors. Up to 90% of CAD risk is preventable by modifying environment (food, exercise and pollution) and it is more common in men than women as well as in Africans than Asians or Europeans. All of these characteristics are consistent with the definition of multifactorial disease (Castelli *et al.* 1986, Doyle *et al.* 1962, Yusuf *et al.* 2004, Kannel *et al.* 1961, Kannel *et al.* 1971, Kannel and McGee 1979, Keys *et al.* 1963, Keys 1980).

Several problems of studying multifactorial diseases are highlighted in table 1.

Table 1. Challenges in multifactorial disease research (Kullo and Ding 2007)

Issue	Description
Heterogenity of phenotype	Multifactorial diseases clinical manifestations are diverse, for example CAD can present itself as stable angina, myocardial infarction, acute coronary syndrome and sudden cardiac death. Also classification can be dichotomous (presence or absence) or continuous (measurements of coronary artery calcification).
Heterogenity of genotype	Genetic heterogeneity is very likely due to different pathways that can lead to multifactorial disease. In case of pituitary adenomas (PA) several monogenic forms are known, somatic mutations in *GNAS* are common in somatotropic PA and several genetic variants are associated with development and characteristics of human PA. Even more genetic heterogeneity is pronounced in CAD, where several CAD risk factors (low-density lipoprotein (LDL) cholesterol, triglycerides) are themselves influenced by genetic variants.
Low influence of individual gene	Single genetic variant usually contributes only tiny proportion towards multifactorial disease phenotype. Low odds ratio (OR) 1.1-1.5 is common contribution of individual genetic variant. To discover such influence large cohorts of cases and controls are necessary which in turn have provided incentive to establish and develop population based genome biobanks in many countries.
Gene-gene and gene-environment interaction	To establish contribution of gene-gene and gene-environment interactions towards risk and pathophysiology of multifactorial disease with sufficient even larger sample sizes are necessary or new methods of quantifying interaction must be developed. Nevertheless it could be important part of "mission heritability" in multifactorial diseases.
Rare causal variants of multifactorial diseases	Majority of genome wide association studies (GWAS) assumed that multifactorial diseases are caused by common genetic variations. Most likely both common and rare variants are involved in development of multifactorial diseases. To discover rare variants ever larger sample size is needed and combination with functional research is necessary.

Another aspect of genetic research of multifactorial diseases is possible use of genetic markers to improve risk prediction and reclassification of patients. Currently multiple studies have focused on use of GWAS data in cardiovascular disorders and diabetes, including complications (meta-analysis (Muller *et al.* 2016)).

The main recommendations as of 2017 of using genetic markers in risk prediction and studies of risk prediction in multifactorial diseases are:

- Evaluation of the baseline model predicting the risk of disease. As baseline model becomes better the harder it is to improve it. This evaluation helps to understand how much improvement of risk assessment can be expected from addition of genetic information(Muller *et al.* 2016).
- Bias avoidance is necessary. Identification of potential sources of bias and negation of its effects must be included in risk prediction study design. The best strategy would be to select and evaluate model in separate, independent training and testing cohorts. Also resampling methods could be used as less powerful substitute (Steyerberg *et al.* 2001).
- Genetic variants included in the risk assessment must be well validated, preferably in several studies across different populations. Information about the effects should be updated regularly as new studies are published modifying current knowledge.
- More studies are necessary to unravel molecular basis of intermediate phenotypes of the multifactorial disease and how these variations relate to the disease in question.
- Models of risk prediction are more likely to be more powerful when weighted risk of the alleles is used than simple counts of risk alleles.
- Evaluation and reporting of more than one quality measure that complement each other should be encouraged. Area under curve (AUC), reclassification measures, recalibration and measures of clinical relevance (number needed to screen) helps to compare various studies, interpret relevance and make decision about potential use of genetic markers (Muller *et al.* 2016).

Meanwhile research of PA also have identified multiple causes of PA and multiple modifiers, that can lead to enhancement of precision treatment strategies of PA not only by stratifying into currently used broad PA type groups but also peering into subdivisions of the disorder (Syro *et al.* 2015).

Research into multifactorial diseases is expected to spark new and advanced possibilities in precision medicine - prevention and treatment of disease that is tailored to individual

variability. This concept is not new, because blood typing to guide blood transfusion is in use for more than century and use of CYP2C19 genotyping is recommended in prescription of anticoagulant clopidogrel ((FDA) 2010). But there is also emerging evidence of precision medicine in its broader scope to provide great results in multifactorial diseases such as cancer (de Bono and Ashworth 2010).

Several hurdles exist for precision medicine, but by discovering how can patient benefit and how much does it cost to healthcare will help to overstep these limitations (Collins and Varmus 2015).

Summary of four peer reviewed publications of genetic research of multifactorial diseases in Latvian population are presented in this work.

1.2 Overview of CAD and its genetics

1.2.1. Anatomy and physiology of cardiovascular system

The cardiovascular system is part of a circulatory system in the humans and other animals with closed (blood never leaves network of blood vessels) cardiovascular system. It consists of heart, three main types of blood vessels (arteries, veins and capillaries) and blood itself. Cardiovascular system has range of functions with most obvious being transport of nutrients (carbohydrates, amino acids, fatty acids, ions and vitamins), oxygen, carbon dioxide, heat, hormones and other signaling molecules as well as cells throughout the body. It is an integral part of immunity necessary for fighting infections. Transport functions also helps to regulate and maintain optimal conditions for cells of the body by stabilizing temperature and pH level (Graaff 2001).

Heart is the central unit of the cardiovascular system by moving blood throughout the body. Arteries and arterioles are muscular and elastic blood vessels that carry blood leaving heart. Their anatomy reflects need to resist pulsating pressure of the blood and deliver it to the organs and tissues of the body Figure 1. It is the arteries that are damaged in case of CAD and their dysfunction leads to pathophysiology of CAD.

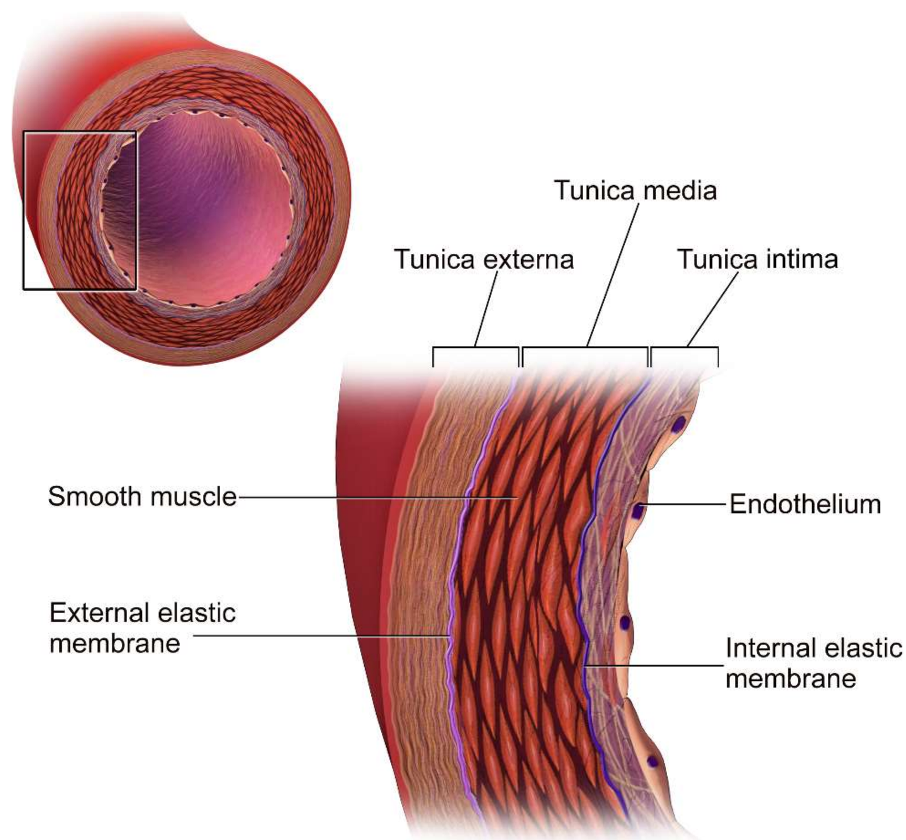

Figure 1. Anatomy of the arterial wall (Blausen 2014).

Capillaries create microvasculature and ensure that every cell of the organism receives nutrients and oxygen, stays connected to the rest of the body and removes waste products of cellular metabolism.

Although heart is filled with blood, oxygen and nutrients cannot be used by heart muscle (myocardium) directly. Coronary circulation delivers these essentials to the myocardium and other components of the heart via coronary arteries. This is an "end circulation" system meaning that there is almost no redundancy and reason why blockage of one vessel is so critically dangerous for whole organism.

Coronary arteries begin at left side of the heart at the base (root) of the aorta. Three aortic sinuses are located in the aortic wall superior to the aortic semilunar valve. Two of these sinuses are beginning of the left and right coronary arteries. Branches of coronary arteries that remain on the surface of the heart are called epicardial coronary arteries, but branches that are inside myocardium are referred as subendocardial coronary arteries. Coronary arteries are self-regulating to maintain optimal blood flow required by the myocardium. If there is lack of oxygen in the myocardium due to decrease of blood flow tissue ischemia occurs. Short term ischemia causes intense chest pain (angina). Prolonged and severe ischemia may result in death of heart or part of myocardium (event known as myocardial infarction) (Graaff 2001). Sudden restoration of blood flow is also dangerous to the cells of myocardium due to creation of reactive oxygen radicals from the anaerobic metabolites (process described as ischemia-reperfusion injury) (Yellon and Hausenloy 2007).

Chronic moderate ischemia leads to myocardial hibernation – condition with weakened heart contractions.

Coronary circulation has unique characteristics in response to different stresses, drugs and hormones. For example, in adrenergic stimulation most blood vessels constrict in response to norepinephrine and blood pressure is increased, but due to relatively higher amount of beta-adrenergic receptors in coronary circulation, coronary arteries dilate and increase blood flow in order to supply heart with nutrients and oxygen in higher workload environment (Graaff 2001).

1.2.2. Coronary artery disease

Coronary artery disease (CAD) (also known as coronary heart disease, acute coronary syndrome, ischemic heart disease) is the most common of cardiovascular diseases (CVD). CAD is usually defined with well-established clinical manifestations such as stable and

unstable angina, nonfatal myocardial infarction (MI), and coronary death. Also CAD can be defined by healthcare procedures such as Coronary artery bypass grafting and percutaneous coronary intervention (including stenting and angioplasty) performed to the patient (Wong 2014).

1.2.3. Mechanisms of CAD

The basis of CAD pathology is atherosclerosis in coronary heart arteries which is characterized with endothelial dysfunction and narrowing of arterial lumen increasing over time. Atherosclerosis itself is mainly driven by inflammation processes at the sites of damaged endothelium (Libby 2002). In this process fatty atheromatous plaques are developing under the endothelium in the arterial tunica intima (located between endothelium and tunica media in the wall of artery) Figure 2.

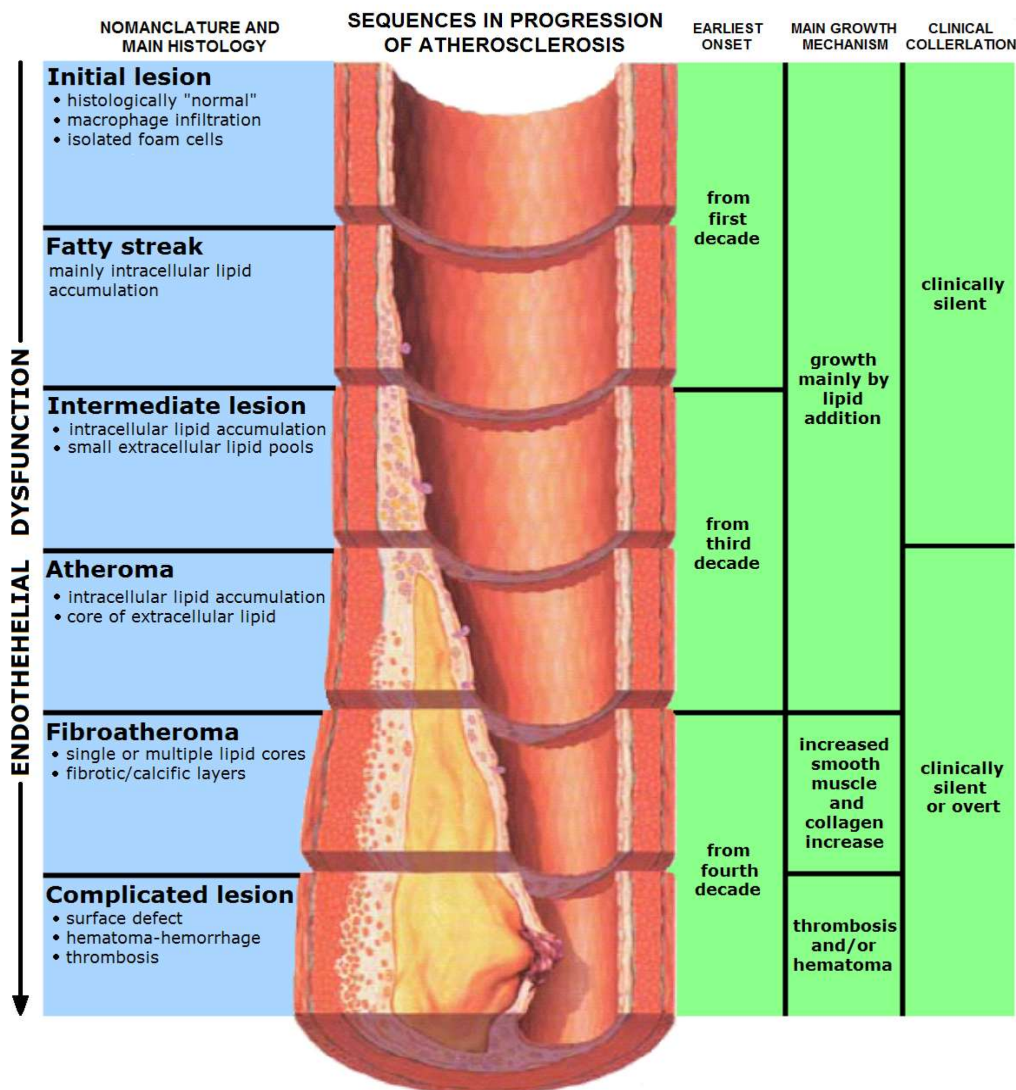

Figure 2. Phases and progression of the atherosclerosis (Anonymous 2006).

Plaque formation is slow, usually continuing over several decades. Interaction between risk factors, cellular events and response to these in arterial wall is the cause of plaques (Libby and Theroux 2005). "Response to the injury" is the main theory currently accepted among scientists and medical scholars about development of atherosclerosis. Plaque formation is triggered by physical and chemical injuries to the endothelial cell layer of arteries (Libby 2002). Main factors causing this injury are physical damage (as a result of direct trauma or hypertension), turbulent blood flow (branching areas of the arteries), circulating reactive oxygen species and pollutant particles (from smoking or air pollution), hyperlipidemia (high blood concentrations of LDL and very low-density lipoprotein), chronically elevated blood glucose and other (Castelli *et al.* 1986, Doyle *et al.* 1962, Kannel *et al.* 1961, Kannel *et al.* 1971, Kannel and McGee 1979, Keys *et al.* 1963, Keys 1980). These factors also predispose plaque formation in certain areas of the arteries (Samady *et al.* 2011).

Endothelial cells in the blood vessel provide semi-permeable barrier between the liquid blood and wall of the blood vessel which is actively involved in several critical roles for the organism. The main function is to regulate exchange of nutrients, gases, water and waste between the blood and tissues. Endothelial cells also regulate diameter of the blood vessels by synthesizing and releasing nitric oxide and prostacyclin (PGI_2) to relax blood vessels and endothelin and angiotensin-II to constrict them. These molecules also help to maintain blood pressure (Lerman and Zeiher 2005).

Endothelium also provides surface that allows blood to flow without sticking to blood vessel walls. When there is an injury to the endothelium inflammatory response is triggered by secretion of cytokines. Also endothelium cells start producing molecules that ensure surface adhesion which causes monocytes and T-lymphocytes to migrate under the endothelium. Endothelial cells near the injured site also change shape and loosen gaps between cells so that monocytes and T-lymphocytes can cross the endothelium. This causes lipoprotein and especially low-density lipoprotein (LDL) particles to enter arterial wall and oxidize due to presence of vasodilator molecule NO, macrophages and lipoxygenase. Additional circulating monocytes are invading injury because of released chemokines (such as IL-8 (Novo *et al.* 2015)), they differentiate into macrophages and start to uptake oxidized LDL. This in turn changes their morphology and in time they are so full of lipids that change appearance to "foam" cells. "Foam" cell phase will trigger apoptosis and it will die, but lipids and debris from all the apoptotic cells will cause inflammation to increase beginning self-driven process of plaque growth in the wall of the artery (Libby 2002).

Meanwhile T lymphocytes secrete cytokines inviting smooth muscle cells to cross internal elastic membrane of the artery wall. These smooth muscle cells start to proliferate due to influence of growth factors. Together with accumulation of lipids, cell debris and calcium deposits smooth muscle cells contribute towards growth of plaques and remodeling of arterial walls. Two types of plaques are found in the wall of arteries: “stable” plaques with relatively small lipid pool and thick fibrous cap, usually preserving lumen of artery, and “vulnerable” plaques characterized with large pool of lipids, partially obstructed lumen, thin fibrous cap and large number of inflammatory cells. Former usually lead to stable atherosclerosis and few clinical CAD signs. Latter type of plaques are more prone to rupture and thrombosis which is especially dangerous in coronary arteries of the heart (Libby 2002).

Stenosis or blockade of blood vessel is event of advanced atherosclerosis, but may never occur to the individual. Complete blockage or 100% stenosis is common cause of the most dangerous CAD endpoints, especially miocardial infarction (Davies 1996).

1.2.4. Risk factors of CAD

The main risk factors of CAD are described as modifiable such as type 2 diabetes, hyperlipidemia, smoking, hypertension and increased C-reactive protein level in the blood. Non-modifiable CAD risk factors are age (above 45 for men and above 55 for women), genetic predisposition and male sex. Low impact risk factors are adiposity, sedentary lifestyle, elevated triglyceride levlel and increased serum homocysteine (Berenson *et al.* 2016, Castelli *et al.* 1986, Doyle *et al.* 1962, Kannel *et al.* 1961, Kannel *et al.* 1971, Kannel and McGee 1979, Keys *et al.* 1963, Keys 1980, Wilcken and Wilcken 1976).

1.2.5. Diagnosis

Two to three decades ago the main focus for diagnosis of advanced CAD was detection of narrowing of arterial lumen using angiography and sometimes physical “stress testing”. But human clinical studies have shown that plaque rich sites with no to low lumen narrowing are the most prone to rupture and cause the occlusion of artery (and life threatening CAD endpoint) within minutes. This is supported by the findings that only about 14% of clinically relevant CAD events are at sites with lumen stenosis over 75%(Maseri and Fuster 2003). Majority are caused by sudden plaque rupture. Therefore currently main emphasis is detection of aterial wall remodelling. Anatomical methods are more precise and allow to monitor disease progression, but require more resources and sometimes are invasive.

Coronary calcium scoring by computer tomography, carotid intimal media thickness measurement by ultrasound, and intravascular ultrasound are anatomical methods that attempt to detect and quantify vulnerable plaques and with subsequent visits monitor CAD progression. Circulating biomarker measurement methods use lipoprotein subclass analysis, glycated hemoglobin or HbA1c, high-sensitivity C-reactive protein, and homocysteine to detect CAD before clinically relevant symptoms are visible. Recent additions of diagnostic techniques are positron-emission tomography and single-photon emission computed tomography imaging to investigate and quantify plaques (Beller and Heede 2011).

1.2.6. Epidemiology

CAD is the most prevalent of CVD and is cause 46% of cardiovascular deaths for men and 38% for women (Wong 2014). And for up to third of the cases the cardiovascular death is the first manifestation of CAD. It is a multifactorial disease which is caused by combination of genetic, environmental and lifestyle factors.

Studies have shown that CAD causative atherosclerosis initiates before age of 20, but usually clinical manifestations appear after several decades. Atherosclerosis increases more rapidly after age of 30 in some cases but more typical age of notable plaques is beyond 50 or 60 years.

The incidence of CAD and the prevalence of important risk factors vary greatly according to geographical region, sex, and ethnic background (Wong 2014).

1.2.7. Historical CAD studies

Already in 1880s there was notion that there is lack of data about heart disease (Hirsch 1883). The first prospective study of heart disease was started in 1946 in Minnesota, USA involving men as the study subjects (Keys *et al.* 1963). Experience and knowledge obtained from the Minnesota studies of CVD in men led to development of The Seven Countries Study and the era of modern CVD epidemiology (Keys 1980). It was proposed that serum cholesterol is cause of CAD and that there is difference in CAD frequency between countries in 1953 (Keys et al. after (Wong 2014)). The Seven Countries Study established basis of the current understanding in CVD (including CAD) risk factors deriving from diet and lifestyle by analyzing data of 12763 men aged 40-69 across multiple geographic regions. The main discovery of the study was that blood cholesterol was directly associated with CVD across all populations involved in the study (Keys 1980).

By this time Framingham Heart Study (FHS) had given its first report that CVD is age and sex related as well that elevated blood pressure, cholesterol levels and obesity are strong predictors of CVD (Dawber *et al.* 1957). Further role of CVD risk factors was elaborated in series of representative publications describing role of cholesterol (Kannel *et al.* 1971), smoking (Doyle *et al.* 1962) and high blood pressure (van den Hoogen *et al.* 2000). FHS also identified negative correlation between high-density lipoprotein and CVD independent of total cholesterol level in the blood (Castelli *et al.* 1986) as well as important link between CVD and diabetes (Kannel and McGee 1979). It demonstrated that several risk factors can be combined further elevating the risk and was the first that developed and introduced CVD risk assessment for the individual (Framingham Risk Score) still in use in clinical cardiology, where it is used as reference in many modern cardiovascular research proposals and comparisons (Wilson *et al.* 1998)

1.2.8. Genetic architecture of CAD

CAD is multifactorial disease therefore risk for each individual is composed of genetic, environmental and lifestyle factors as well as their combination and interaction (Khera *et al.* 2016) CAD heritability was supported by clinical observations in the middle of 20th century (Gertler *et al.* 1951) and further confirmed by study of 20000 Swedish twins which estimated heritability for fatal CAD of ~50% (Marenberg *et al.* 1994, Zdravkovic *et al.* 2002). Using current genome wide data and updated methods CAD heritability was estimated 40-50% (Won *et al.* 2015) which is similar to the Swedish twin study. FHS also found that family history of cardiovascular disease in a parent or sibling was severely increasing risk of CAD incidence (Lloyd-Jones *et al.* 2004, Murabito *et al.* 2005).

These were the pioneering studies that started research in genetics of CAD, understanding molecular mechanisms of the disease and how to translate them into clinical practice (Khera and Kathiresan 2017).

Historically three types of research methods mostly have been used to unravel details about genetic details underlying CAD in humans. Family linkage studies, where relatives with and without CAD across several generations had their genome mapped and causative locus (preferably) identified. Candidate gene studies did research of one to several genes or genetic variations (based on hypothesis about plausible involvement of gene or variant in CAD) using groups of unrelated individuals (cases and controls). Latest research focuses on whole genome approach investigating either hundreds of thousands of genetic variants

across genome or sequencing whole or large parts (usually exome) of the genome. Latter is hypothesis free approach with aim to find all genetic risk factors and estimate their contribution towards development of CAD (Franchini *et al.* 2008)

Information from genotyping is used to investigate association between phenotype and genotype. Association basis is difference of observed allele frequency between case and control groups. Results usually are reported as odds ratio (OR) with confidence interval (industry standard is 95%). Control group is reference, therefore OR above one describes genetic variant with increased relative risk, but less than one – decreased relative risk (Franchini *et al.* 2008).

CAD end and intermediate phenotype is multifactorial and many genetic variants can lead to similar clinical endpoints. Wide range of genetic variants is responsible for increased risk and susceptibility of CAD. Some are located in genes of receptors, structural proteins and enzymes of metabolic pathways (regulating blood pressure, lipid metabolism, coagulation, growth and inflammation), while most of the latest studies have found strongest associations in intergenic regions (Nikpay *et al.* 2015).

1.2.9. Candidate gene studies

Hundreds if not thousands of candidate gene studies have been performed in search of genetic risk factors of CAD across many populations and racial backgrounds.

Initially candidate gene studies were using relatively low number of cases and controls and those were predominantly of European descent. Results were rarely replicated and many studies attempting to replicate previous findings were prone to lack of power themselves (Morgan *et al.* 2007).

The first attempt to replicate large amount of previous discoveries of associations of candidate genes with CAD were published in 2007. It was focusing on studies published before March 10, 2005. This study tested 85 variants in 70 genes in 811 cases and 650 age- and sex-matched controls. Study group was European descent. Results revealed that only one out of 85 genetic variants show significant association before correction for multiple testing. Moreover just 41 (48.2%) of designated risk alleles were more frequent in cases than in controls while one was completely identical (Morgan *et al.* 2007).

But some controversy remains as this study failed to validate variants that were replicated in other studies. *APOEε*4 allele was replicated as increasing risk of CAD (Song *et al.* 2004). Also *THBS1*, *THBS2* and *THBS4* were associated with CAD in publication of Topol *et al.* (Topol et al.

2001) and *THBS1* replicated by (Zwicker *et al.* 2006) as risk factor with early (before age 45) myocardial infarction. While other studies especially GWAS have failed to demonstrate association (Morgan *et al.* 2007, Nikpay *et al.* 2015) or showed trend of association with early CAD in case of *THBS2* and *THBS4* (McCarthy *et al.* 2004).

Recently there have been upsurge of large, well designed candidate gene studies in CAD in populations (East Asian in particular) that are not European descent which were dominating CAD candidate gene studies in late 20th and first decade of 21st centuries (Jin *et al.* 2014, Zhang *et al.* 2013, Zhang *et al.* 2017).

1.2.10. Family lnkage studies

Family linkage studies are very successful in discovery of basis of monogenic diseases and it was hypothesized that they will be as insightful for CAD. Family linkage studies use knowledge of Mendel's laws of inheritance that propose equal distribution of genetic markers within family. It investigates whole genome for markers that are associated with presence of disease. Then fine mapping is performed with expectation to find causal gene. Main family linkage study results in CAD studies are shown in table 2. Yet like candidate gen studies replication of results proved difficult despite high quality design of the studies. Also fine mapping of the locus often were not enough to narrow down causal genetic variation or even gene (Franchini *et al.* 2008).

Table 2. Main familial linkage studies of CAD (based on (Franchini *et al.* 2008)).

Study author	Year	Population screened	Study sample	Clinical event	Chr locus	LOD score	Gene identified
Pajukanta	2000	Finland	156 families (344 indiv)	Early CAD	2q21-22 Xq23-26	3,7 3,5	-
Francke	2001	Mauritius	99 families (525 indiv)	CAD	16p13	3,06	-
Broeckel	2002	Germany	513 families (1406 indiv)	Early CAD	14	3.9	-
Harrap	2002	Australia	61 families (161 indiv)	CAD	2q36	2,6	-
Wang	2003	USA	1 family (25 indiv)	CAD or MI	15q26	4,19	*MEF2*
Helgadottir	2004	Iceland	296 families (2454 indiv)	MI	13q12-13	2,86	*ALOX5AP*
Hauser	2004	USA	238 families (1168 indiv)	Early CAD	3q13	3,3	-
Samani	2005	United Kingdom	1933 families (4175 indiv)	CAD /MI	2q14-21	1,15	-
Helgadottir	2006	Iceland	296 families (2454 indiv)	MI	12q22	NZ	*LTA4H*
Farral	2006	Europe	2036 families (2658 indiv)	CAD /MI	17q21	2,68	-

1.2.11. GWAS

Research of CAD genetics using GWAS approach started in the early 21st century and the first results were published in 2002 by Ozaki et al. showing association of five *LTA* single nucleotide polymorphisms (SNPs) with MI in Japanese population. Genotyping "only" 93000 SNP it was a frontrunner of a modern GWAS era. Functional research of missense SNP (Thr26Asn) in *LTA* revealed doubling of synthesis of adhesion molecules in smooth muscle cells of the blood vessels including coronary heart arteries. Another SNP in the first intron of the *LTA* increased its transcription (Ozaki *et al.* 2002). Yet this and some other early GWAs discoveries were not replicated later in larger and more powerful studies (Table 3).

As of 2017 about 100 loci across human genome have been associated with CAD (MacArthur *et al.* 2017) (Table 3).

Table 3. Main GWAS studies of CAD (MacArthur *et al.* 2017).

Author	Year	Population	Sample cases/controls	Validation sample	New CAD loci	Replicated previously
Samani	2008	European	1926/2938	875/1644	7	0*
WTCCC	2008	European	1926/2938	875/1644	5	0*
Helgadottir	2008	European	1607/6728	2980/6309	1	0*
Erdmann J	2009	European	1222/1298	18.2k/20.0k	2	0
Kathiresan	2009	European	2967/3075	9746/9746	5	4
Tregouet	2009	European	1926/2938	7073/7325	1	0
Reilly	2011	European	1808/915	10585/6468	2	2
Lettre	2011	African American	260/5053	621/1629	3	0
Erdmann J	2011	European	1157/1748	7887/8244	1	0
Aoki	2011	East Asian	194/1539	5177/6220	1	0
Schunkert	2011	European	22.2k/64.8k	56.7k/56.7k	18	13
C4D	2011	European/ South Asian	8424/7268 6996/7794	18.0k/16.4k33 59/2828	9	10
Wild	2011	European	2078/2953	19.4k/35.4k	1	4
Slavin	2011	European	2000/3000	-	4	1
Mehta	2011	European/South Asian	15.4k/15.0k	21.4k/19.1k	0	2
Takeuchi	2011	East Asian	806/1337	3593/6335	1	2
Dawies	2012	European	7123/6826	5211/5821	5	0
Hager	2012	Middle Eastern	1949	2547	0	1
Lu	2012	East Asian	1515/5019	15,5k/11.5k	4	4
Lee	2013	East Asian	2123/3591	3052/4976	1	4
Hirokawa	2014	East Asian	1666/3198	11.4k/28,4k	2	2
Nikpay (CAD)~	2015	Mixed (European)	61.3k/126.3k	-	15	30
Nikpay (MI)~	2015	Mixed (European)	38.1k/126.3k	-	8	30
Dehghan (CAD)~	2016	European	2406/21.6k	3059/28.1k	1	1
Dehghan (MI)~	2016	Mixed (European)	1570/21.6k	2874/4.9k	3	1
Wakil	2016	Middle Eastern	2563/2266	503/371	7	11

CAD – coronary artery disease, WTCCC – Wellcome Trust Case Control Consortium, k – thousands, C4D – Coronary Artery Disease Genetics Consortium, MI – myocardial infarction, * three initial CAD GWAS studies reported 9p21.3 locus at the same time. ~reported CAD

and MI as separate endpoints. Comprehensive CAD GWAS summary can be found in Supplementary 5.

One of the earliest and most consistently replicated across multiple populations is 9p21 locus, associated with CAD or MI in more than ten populations and counting (Supplementary 5). Latest CAD GWAS are focused on meta-analysis of many studies and therefore have increased in size significantly. If sample size in 2005-2008 were 1000 to about 4500 for cases, then latest meta-analyses are using over 60 000 cases and 120 000 controls (Table 3) (MacArthur *et al.* 2017, Nikpay *et al.* 2015). That has had great effect on study power and ability to discover ever smaller effects on development of CAD. Early associations had OR range about 1.20-2.10 per SNP. One of latest studies are reporting ten novel variants associated with CAD with OR ranging from 1.06 to 1.20 under additive model (Nikpay *et al.* 2015). Same authors report interesting and by nowadays standards strong association (OR 1.5) of SNP rs12976411 located in chr19 non-coding RNA (*LOC400684*) protecting from CAD under recessive model, but no association under additive model (Nikpay *et al.* 2015). This way of testing CAD risk is certainly underreported and hard to estimate what % of studies have performed such tests and not reported insignificant associations.

Variants associated with increased CAD risk can be divided into groups based on their mechanism of action which contributes to development of CAD. These groups describe known conventional risk factors. They are LDL cholesterol, triglycerides, blood pressure, inflammation, plaque formation/vascular remodeling, nitric oxide/cyclic guanosine monophosphate signaling and large group of “others” where possible involvement in CAD risk increase is still unclear.

One of prominent groups of increased genetic and also conventional risk factors of CAD is linked with non-optimal LDL regulation. Increased blood LDL level (above 1.8 mmol/L) is known CAD risk factor and lipid-lowering therapy (by using class of drugs called statins or HMG-CoA reductase inhibitors) is employed to prevent and or delay CAD symptomatic onset. LDL receptor (LDLR), encoded by *LDLR* found in humans at chr19p13.2 locus, is important regulator of LDL metabolism (Cyrus *et al.* 2002). LDLR in liver cells uptakes circulating LDL for internal use and lowers its level in the blood (Zhang *et al.* 2013). Also LDLR can recognize apoE protein from chylomicrons and very low-density lipoprotein particles (Schmidt *et al.* 2008). Dysfunctional LDLR or its regulation is often causing of familial hypercholesterolemia (Benlian *et al.* 1990, Soutar and Naoumova 2007). GWAS have identified *LDLR* variations with increased CAD risk (Bis *et al.* 2011, Myocardial Infarction

Genetics *et al.* 2009) and pro-atherosclerotic lipid profile (Myocardial Infarction Genetics *et al.* 2009, Sarwar *et al.* 2007). The T allele of rs2228671 is associated with decreased LDL concentration and with decreased CAD risk, while T allele of rs688 is associated with increased LDL concentration and mRNA splicing efficiency (Jamaldini *et al.* 2014). Also a separate study has found that rs688 is important for proper endosomal recycling of LDLR (Gao *et al.* 2013). CAD risk is also increased in carriers of rs1122608 G (more common) allele (Kullo and Cooper 2010, Myocardial Infarction Genetics *et al.* 2009, Zhang *et al.* 2013) OR 2.09 (CI 0.95 1.48–2.97) ranging from OR 1.82 in combined East Asian populations to OR 2.31 in Europeans to 3.37 in East Asian males (Liu *et al.* 2016). This variant is located at 58.7 kilobases (kb) upstream of the *LDLR* gene. It has been associated with CAD risk under dominant model in European populations, but studies in East Asian populations show mixed results (Yang *et al.* 2010, Wang *et al.* 2014, Zhang *et al.* 2013) which meta-analysis attributed to gender difference (G allele is a risk factor for East Asian men, but not women) (Liu *et al.* 2016). At least two studies have reported *LDLR* association with CAD independently from lipid profile of the patients (Jamaldini *et al.* 2014, Martinelli *et al.* 2010).

One of the mechanisms of variations associated with CAD risk involves triglycerides and multiple variations in different genes and is centered around enzyme lipoprotein lipase (LPL). LPL reduces triglyceride level in the blood by hydrolyzing lipoprotein-bound triglycerides. Its activity is upregulated by apolipoprotein A-V (APOA5), but reduced by apolipoprotein C-III (APOC3) and angiopoietin-like 4 (ANGPTL4). Variants in genes of these proteins have been associated with CAD in GWAS. Missense variant (p.D36N; minor-allele frequency, 1.9%) in *LPL* leads to 20% reduced enzyme activity and is associated with increased CAD risk (Myocardial Infarction *et al.* 2016). *ANGPTL4* missense variation p.E40K leads to inactive ANGPTL4 (negative LPL regulator) and is cardio-protective due to significantly reduced triglyceride levels while not influencing LDL or high-density lipoprotein (Myocardial Infarction *et al.* 2016). At the same time mutations in positive LPL regulator APOA5 increases plasma triglycerides and risk of CAD (Do *et al.* 2015). And in line with *ANGPTL4* negative LPL regulator's *APOC3* loss of function variants also are cardio protective (Tg *et al.* 2014). This detailed knowledge (Figure 3) of inner regulation of LPL system has led to the identification

of novel drug targets and APOC3 inhibitors are already in development and clinical trials (see chapter 1.6).

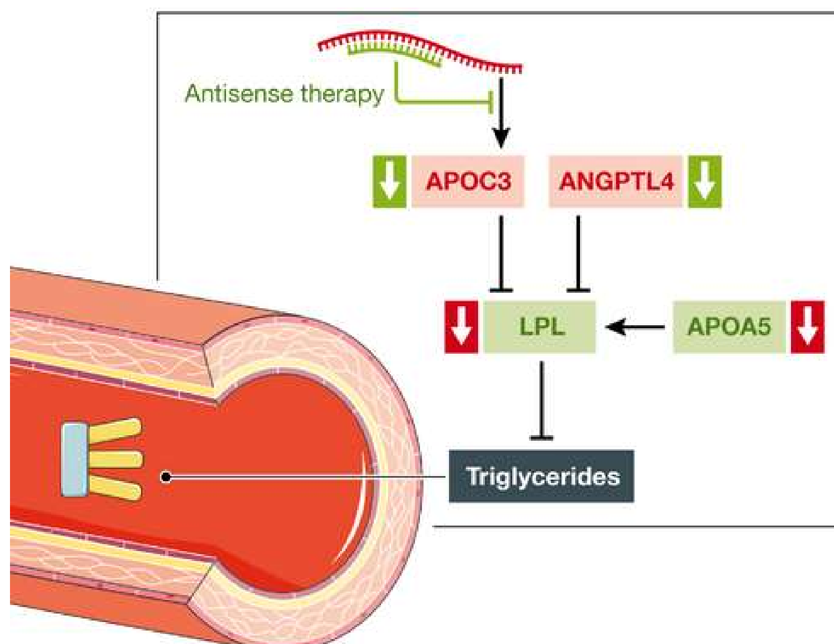

Figure 3. Regulation of blood triglyceride level by LPL and its regulators (Kessler *et al.* 2016).

9p21 deserves special mention due to world-wide reproduction of association results and puzzling role of this locus how DNA variations are translated into pathophysiology. Association of 9p21 with CAD and MI was discovered in 2007 by four independent research groups (Helgadottir *et al.* 2007, McPherson *et al.* 2007, Samani *et al.* 2007, Wellcome Trust Case Control *et al.* 2007). Risk allele homozygotes had observed CAD OR from 1.20 to 1.37 (CI 0.95 1.02-1.58) in three studies (McPherson *et al.* 2007, Samani *et al.* 2007, Wellcome Trust Case Control *et al.* 2007) and MI OR 2.02 (CI 0.95 1.72-2.36) in the fourth (Helgadottir *et al.* 2007). The locus is 58kb in size and multiple strong association signals have been observed (Helgadottir *et al.* 2007, McPherson *et al.* 2007, Samani *et al.* 2007, Wellcome Trust Case Control *et al.* 2007). Follow up studies and meta analyses confirmed the association in various populations (Cakmak *et al.* 2015, Chan *et al.* 2011, Cheng *et al.* 2011, Foroughmand *et al.* 2015, Kumar *et al.* 2011, Meng *et al.* 2008, Nawaz *et al.* 2015). Also associations with various other cardiovascular conditions (Yamagishi *et al.* 2009, Niemiec *et al.* 2017, van 't Hof *et al.* 2016) as well as diabetes (Cheng *et al.* 2011, Cugino *et al.* 2012) and cancers have been reported (Li *et al.* 2014, Merlo *et al.* 1994). Fine mapping of the region was unable to pinpoint causal mutation (Congrains *et al.* 2012). Nearest genes are at least 90kb away and are known tumor suppressors *CDKN2B* and *CDKN2A* (Cunnington and

Keavney 2011). Also main association cluster is next to long non-coding RNA, *ANRIL*, which has multiple transcripts and CAD associated variants could alter its expression. For some time *ANRIL* was the main suspect of CAD causality (Congrains *et al.* 2013, Pasmant *et al.* 2011)until allelic imbalance and expression quantitative trait loci studies showed that 9p21 contains enhancers which are the main regulators of *CDNK2B*, *CDNK2A* and *MTAP* expression. That would mean that all genes in this locus contribute to CAD susceptibility (Cunnington *et al.* 2010, Liu *et al.* 2009, Motterle *et al.* 2012), but several studies have failed to replicate finding and leaving question open to debate and new research (Congrains *et al.* 2012, Holdt *et al.* 2011, Jarinova *et al.* 2009, Pilbrow *et al.* 2012). *ANRIL* is conserved among primates, but not in mice hampering to understand its role in CAD (Jarinova *et al.* 2009) but when 9p21 homology locus were deleted in inducible knockout mice model, expression of both *Cdkn2a* and *Cdkn2b* were decreased supporting hypothesis of enhancer region at 9p21 (Visel *et al.* 2010).

GWAS discoveries have led to expansion of computational genomic methods in order to untangle complexity of CAD risk factors. Gene-gene, gene-environment interactions as well as epistasis between multiple alleles may be responsible for non-linear increase in CAD risk allowing for “missing heritability” to exist even within already discovered susceptibility variations (Nikpay *et al.* 2015, Zuk *et al.* 2012). Also integration of disease-related data from functional genomics allows to reduce complexity of finding “true” causal variant (Kichaev *et al.* 2014, Trynka *et al.* 2015). Bayesian networks can be used in key driver analysis to find driver genes in CAD (Makinen *et al.* 2014) and together with protein-protein interaction and co-expression data Bayesian networks have identified *TNFRSF13C* and *EBF1* as CAD susceptibility genes (Huan *et al.* 2013). Also, in case of 9p21, chromosome conformation capture method was used to investigate pairwise interactions between *CDKN2B*, *CDKN2A*, *MTAP* and *INF2A* and it was discovered that interactions were modulated by INFγ (Harismendy *et al.* 2011).

Additionally, whole exome sequencing in high risk individuals and confirmed healthy controls have shown possibility to identify rare variants protecting coronary arteries from formation of plaques (Abramowitz *et al.* 2016).

These examples demonstrate that integration of data from various biological levels (genetic from GWAS or whole exome sequencing or whole genome sequencing, epigenetic, transcriptomic and proteomic) can lead to more complete picture of a complex system (Hawkins *et al.* 2010, Kwon *et al.* 2012).

1.2.12. CAD genetic risk and its use in clinical setting

Latest trend in GWAS data use is increasing precision of risk scoring for patients. After initial excitement "GWAS: key to personalized medicine" ended, several studies were negative about possibility to use such data to increase resolution of patient classification (de Haan *et al.* 2012, Ripatti *et al.* 2010). Nevertheless, seven out of 17 studies using genetic variants associated with CAD, found statistically significant increase in risk prediction (AUC increase when including genetic information). Nine studies observed non-significant AUC increase in their analysis (Muller *et al.* 2016). Amount of AUC increase can be called modest at best ranging from 0.001 (Tikkanen *et al.* 2013) to 0.07 (Bolton *et al.* 2013). Yet for global healthcare that would mean thousands of people saved because of improved risk prediction and improved targeted treatment (Tikkanen *et al.* 2013). Since nowadays the whole genome genotyping cost is getting comparable to some biochemical analyses of the blood, it offers good cost-benefit ratio to be performed.

For some groups of patients' inclusion information of genetic variations in risk calculation is more beneficial than for others. For example, in group of middle aged men have shown greatest risk prediction improvement in at least two studies (de Vries *et al.* 2015, Hughes *et al.* 2012). Also it seems that calculating weight for each variation is important as studies performing such calculation have higher improvements in patient discrimination and reclassification (Ganna *et al.* 2013). Also using genetic variations that are not associated with intermediate phenotypes of traditional risk factors but directly to CAD increase clinical value of risk prediction especially for individuals with intermediate risk (Lluis-Ganella *et al.* 2012). Inclusion of genetic information in primary CAD prevention in patients at medium risk would avert one CAD event for every 300-400 participants of the screening. That translates to 3.15 (CI 0.95 -1.30-7.60) CAD event free years per 1000 people of general population (Ganna *et al.* 2013). But study involving South Asian population failed to find link between genetic risk score and CAD events and reclassification. Although study design and sample size issues were mentioned main suggestion were that different set of CAD risk variations may be required for accurate risk prediction in the South Asian population (Beaney *et al.* 2015). Probably genetic risk factors should be determined using population of similar ancestry, but most of current genetic CAD risk factors are discovered in studies using populations of European descent (Supplementary 5).

1.2.13. Drug targets trials due to CAD genetics studies

Inhibition of *APOC3* by RNA interference-mediated knockdown is currently being investigated in clinical trials. In a dose-ranging phase II study, ISIS 304801, an antisense *APOC3* inhibitor, led to a dose-dependent 31–71% reduction in triglycerides (Gaudet *et al.* 2015).

Currently investigated *PCSK9* inhibitors exploit monoclonal antibodies to inhibit the effect of *PCSK9* in the circulation. Also RNA interference- and small molecule-based approaches are in the development and evaluation (Fitzgerald *et al.* 2014, Robinson *et al.* 2015).

The development of so-called *precision medicine* is a major goal of current CAD funding concepts (Jameson and Longo 2015). Unfortunately, there is currently only limited evidence to suggest that genetic testing of individual common risk variants allows for stratification into different treatment modalities. Exception however are individuals with a high genetic risk score who appear to have a larger benefit from statin treatment than those with low genetic risk (Hughes *et al.* 2012, Mega *et al.* 2015, Schunkert and Samani 2015).

1.2.14. Adenosine system and CAD

Adenosine receptors (AR) are a group of four known G protein-coupled receptors enabling adenosine signaling within an organism. Their molecular structure is similar to other G protein-coupled receptors with seven α-helical transmembrane domains and an extracellular amino-terminus and an intracellular carboxyl-terminus (Fredholm *et al.* 2011).

Adenosine is purine nucleoside primarily used in modified form to store genetic information and as energy exchange molecule in all living cells. Additional important role of adenosine is signaling and coordination of processes within body. It is produced from metabolism of adenosine triphosphate (ATP) (Okada *et al.* 2006, Picher *et al.* 2003, Robson *et al.* 2005) and have range of functions carried out via four types of adenosine receptors (A1R, A2AR, A2BR and A3R) (Fredholm *et al.* 2011).

Functions of ARs are: modulation of neurotransmitter release (Sebastiao and Ribeiro 2000), synaptic plasticity (Costenla *et al.* 2001) and neuroprotection in ischemic, hypoxic and oxidative stress events (Cunha 2001, Ferreira and Paes-de-Carvalho 2001).

Adenosine produces either vasoconstriction or vasodilation of veins and arteries (Li *et al.* 1998). Adenosine regulates T cell proliferation and cytokine production (Hasko and Pacher 2008). The nucleoside also inhibits lipolysis and stimulates bronchoconstriction (Van der

Graaf *et al.* 1999). Reports in 2016 also highlight its role as non-narcotic analgesic agent (Janes *et al.* 2016).

ARs are classified based on their interaction with adenylyl cyclase to regulate cyclic adenosine monophosphate production. The A1R and A3R are coupled to $G_{i/o}$ protein and reduce activity of adenylyl cyclase (Cunha 2001, Fredholm *et al.* 2011, Paes-De-Carvalho 2002), but A2AR and A2BR are coupled to $G_{s/olf}$ (Fredholm *et al.* 2011) and enhance cyclic adenosine monophosphate production.

A3R is located on chromosome 1 at p13.2 locus. It spans 4.33kb and most transcripts contain one intron (Yates *et al.* 2016).

A3R is mostly studied due to its protective action in cardiac ischemia. Several studies have shown that the A3R is important mediator of cardioprotection during and following ischemia-reperfusion (Headrick and Peart 2005).

There is no agreement how exactly A3AR cardioprotection timing works. A number of studies have indicated that pre ischemic activation of A3R is necessary or crucial (Black *et al.* 2002), but others have argued that main protection is post ischemia (Jordan *et al.* 1999, Maddock *et al.* 2002, Suleiman *et al.* 2001), and also there is articles stating that A3R agonism is cardioprotective with either pre- or post-ischemic treatment (Headrick and Peart 2005). In animal models suggested injury inducers are neutrophils, whose activation is reduced upon A3R agonist administration (Jordan *et al.* 1999). Molecular mechanism also is suggested as A3R induced blockade of mitochondrial permeability transition pore (mPTP) opening, because studies state that mPTP opening is crucial for the ischemia-reperfusion injury to develop (Suleiman *et al.* 2001, Weiss *et al.* 2003). Also a study has reported A3AR activation causes activation of PI3K/Akt pathway which in turn inhibits glycogen synthase with following blockage of mPTP opening (Park *et al.* 2006).

In line with cardiovascular epidemiology and cardioprotection by A3R Ashton et al. has reported decreased mRNA of A3R and increased mRNA of A2BR when aging (Ashton *et al.* 2003). Also decreased amount of A1R has been observed in aged hearts during ischemia which leads to hypothesis that both decreased A1R and A3AR expression is responsible for different results in cardioprotection timing (Borea *et al.* 2009). This hypothesis is expanded in a review that all AR subtypes are involved in cardioprotection, but with different types of cordioprotective modulation and differently during aging and with interplay between each of them (Nishat *et al.* 2016, Philipp *et al.* 2006, Solenkova *et al.* 2006).

1.3 Glyoxalase system in Diabetes mellitus complications

1.3.1. Glycation, methylglyoxal and glyoxalase system

The glyoxalase system with its rate limiting enzyme glyoxalase 1 (GLO1, Enzyme Commission 4.4.1.5.) protects living cells from production and accumulation of advanced glycation end products (AGEs) which are damaging to life-enabling metabolic processes.

GLO1 was discovered in 1913 (Dakin 1913) and more than 100 years of research has provided insight about its critical role in almost all life forms.

The main contributor towards creation of AGEs is a byproduct of glycolysis and other metabolic reactions a toxic compound called methylglyoxal (MG). Other reactive molecules such as glyoxal and 3-deoxyglucosone are minor contributors.

Glucose is main source of energy for the cells. But not all glucose molecules can successfully finish energy production process and some of them forms MG (Phillips and Thornalley 1993). Because glycolysis is occurring in all cells, MG is created in both normal and pathological conditions.

Both enzymatic and non-enzymatic reactions produce MG and it forms at various speeds in various organisms, tissues, cells under different physiological conditions (Thornalley 1996). MG formation via the fragmentation of the triosephosphates glyceraldehyde-3-phosphate and dihydroxyacetone phosphate was discovered in 1993 (Richard 1993).

Up to 0.4% of glycosis molecules in energy metabolism result in MG production (Kalapos 2008). And is elevated when concentrations of precursors of MG are high, as in hyperglycemia, impaired glucose utilization and triosephosphate isomerase deficiency (Ahmed *et al.* 2003). Half-life of MG is short in a biological environment (Kalapos 2008) and it readily reacts with lipids, nucleic acids and with lysine and arginine residues of proteins to form AGEs (Rabbani and Thornalley 2012, Thornalley 2005, Thornalley 2007).

GLO1 of glyoxalase system is the main enzymatic metabolizer of MG and glyoxal (Thornalley 1993). Glyoxalase system together with aldoketo reductases (AKRs) and aldehyde dehydrogenases (ADHs) metabolizes more than 99% of MG providing efficient defense mechanism against formation of AGEs. With the exception of renal medulla GLO1 is more than 30 fold more active than AKRs and ADH (Rabbani *et al.* 2016). GLO1 is both efficient and abundant enzyme in the human cells amounting up to 0.02% of the total protein (Larsen *et al.* 1985). It is known that GLO1 does not metabolize 3DG and its removal is mostly carried out by AKRs and ADHs (Collard *et al.* 2007).

Detoxification of MG is a three step process. At first reduced glutathione (GSH) reacts with MG in non-enzymatic reaction forming hemithioacetal which is further converted by GLO1 to S-D-lactoylglutathione. Hydroxyacylglutathione hydrolase converts this compound to D-lactate and recycles GSH (Figure 4) (Thornalley 1993).

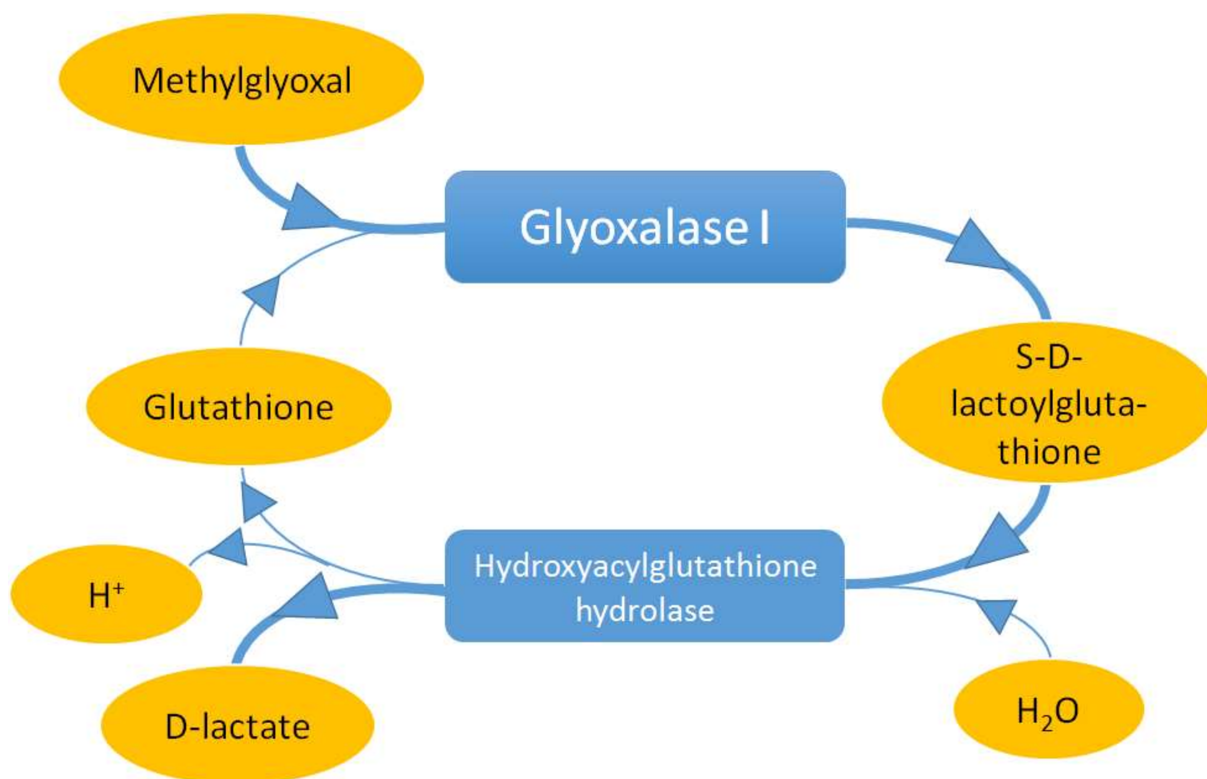

Figure 4. Methylglyoxal detoxification pathway by glyoxalase I and hydroxyacylglutathione hydrolase.

S-D-lactoylglutathione is nontoxic therefore its creation by GLO1 is crucial step for MG detoxification. It can be concluded that GLO1 activity indirectly determines MG toxicity to the cell and amount of AGEs formed. It must be noted that GSH recycling occurs in the last reaction and high amount of MG or low activity of hydroxyacylglutathione hydrolase can lead to GSH deficiency for other cell processes (Dringen 2000).

Glyoxalase system is ubiquitous to almost all organisms (except certain protozoa (Sousa Silva *et al.* 2012)). It is located in cytosol and mitochondria (Thornalley 1990).

Toxicity of MG can be understood and importance of GLO1 can be appreciated when looking on effects of AGEs within cell and whole organism. AGEs are implicated in wide range of pathophysiological processes like ageing (Ahmed *et al.* 2003, Morcos *et al.* 2008), vascular remodeling (Hanssen *et al.* 2014), Alzheimer’s disease (Angeloni *et al.* 2014), autism (Maher 2012), hyperalgesia (Koivisto *et al.* 2014), inflammation (Tikellis *et al.* 2014) and most notably diabetes and its complications (nephropathy (Rabbani *et al.* 2007), neuropathy

(Bierhaus *et al.* 2012, Skapare *et al.* 2013) and retinopathy (Berner *et al.* 2012, Miller *et al.* 2010)). Mechanism how these pathological phenotypes arise from changes in molecules is not always clear although molecular workings and reaction chains within cells are well described.

MG induced glycation often forms complex and stable adducts (Lo *et al.* 1994). These are known as Maillard reactions and start with formation of Shiff's base adduct when MG reacts with lysine and arginine side chains of the protein (Padayatti *et al.* 2001). Typical AGE that forms this way is argpyrimidine, compound with a suspected role in apoptosis (Antognelli *et al.* 2014, Sakamoto *et al.* 2002). Common occurrence is modification of blood serum proteins under normal physiological MG concentrations, but chronically elevated MG levels leads to irreversible change in protein structure. Long lived proteins are at the most risk to form AGEs. MG readily glycates albumin, hemoglobin, mitochondrial proteins and transcription factors (Lo *et al.* 1994, Rabbani and Thornalley 2012). Accumulation of AGEs increases with age resulting in widespread protein damage. MG not only directly influence protein function by amino acid modifications but also these modified proteins interact with AGE receptors (Grillo and Colombatto 2008). Such interaction switches on inflammatory response adding to damage done by MG. It has been shown that MG increase levels of free radicals in dose dependent manner and initiates production of pro-inflammatory factors (TNFα, IL-6 and IL-8) (Desai *et al.* 2010).

Glycation of DNA by MG also has been observed especially for adenine and guanine bases (Thornalley 1996). Not only nucleotide AGE formation causes multibase deletions and substitutions (Murata-Kamiya *et al.* 2000) but also multipolymer crosslinking (DNA-DNA, DNA-protein, protein protein)(Murata-Kamiya and Kamiya 2001, Tu *et al.* 2013) which could be mechanism behind DNA replication inhibition by MG (Kalapos 1994).

Similar to many proteins, GLO1 activity is regulated both by expression and by modification after translation. The first exon of GLO1 contains antioxidant-response element (ARE) (Xue *et al.* 2012) which negatively affects GLO1 expression when hypoxia inducible factor 1α HIF1α binds to it (under hypoxia) (Zhang *et al.* 2012). Also erythroid 2-related factor 2 (Nrf2) decreases GLO1 expression as a stress response (Xue *et al.* 2012). Nrf2 itself is suppressed by nuclear factor kappa-light-chain-enhancer of activated B cells in diabetes, metabolic syndrome and obesity due to chronic hyperglycaemia and inflammation (Bierhaus *et al.* 2001, Liu *et al.* 2008). Other regulatory elements in GLO1 are MRE (metal-response

element), IRE (insulin-response element), E2F4 (early gene 2 factor isoform 4), and AP-2α (activating enhancer-binding protein 2α) (Rabbani *et al.* 2014).

1.3.2. Glyoxalase system in diabetes complications

Hampered GLO1 activity in diabetic conditions (Bierhaus *et al.* 2001, Liu *et al.* 2008)is part of the reason why microvascular complications (nephropathy, retinopathy and neuropathy) are developing in most of the diabetic individuals (Feldman 2003) (McLellan *et al.* 1994, Ratliff *et al.* 1996, Thornalley *et al.* 1989). It has been shown that GLO1 activity is decreased and protein derived AGEs concentration is increased in renal, retinal and neural models of blood vessel complications (Barati *et al.* 2007, Bierhaus *et al.* 2012, Karachalias *et al.* 2010, Miller *et al.* 2010, Palsamy and Subramanian 2011). Cell cultures of vascular endothelial cells in high glucose environment show elevated dicarbonyl stress from increased MG concentration (Dobler *et al.* 2006).

Diabetic retinopathy is mostly caused by damages to blood vessels of the retina and is the main cause of adult blindness in the US. Nine out of ten diabetics have some form of retinopathy within 15 years after diagnosis. Diabetes causes 25 000 cases of blindness a year in the US (Fong *et al.* 2003, Gardner *et al.* 2002). MG increase has been detected in diabetic kidney (Geoffrion *et al.* 2014). Diabetic nephropathy accounts for 40% of new kidney failure diagnoses (Skyler 2001). Neuropathy and painful neuropathy are common long term diabetic complications with critical impact on quality of life of the patient. Painful neuropathy occurs around 10% of cases and pathophysiology is unclear and symptoms often cannot be treated with nonsteroidal anti-inflammatory drugs or opiates (Calcutt 2002). MG concentration in plasma is increased in type 2 diabetes (T2D) patients with painful neuropathy. These findings were strengthened by results in animal model that MG can induce primary hyperalgesia showing potential reason of painful diabetic neuropathy (Bierhaus *et al.* 2012). Activity of GLO1 was significantly negatively correlated ($r = -0.261$, $p = 0.007$) with duration of T1D and was reduced in patients of both types of diabetes with severe painful neuropathy compared to individuals with mild or moderate symptoms (Skapare *et al.* 2013). Most of non-traumatic amputations are caused by diabetic neuropathy. (Feldman 2003)and lifetime risk of amputation for diabetic is around 15% (Feldman 2001). Diminished MG detoxification and following oxidative stress in the microvasculature is significant factor leading to complications in diabetes (Obrosova 2003).

Blood MG concentration was increased up to 5-6 fold in case of diabetes patients (McLellan *et al.* 1994). Elevated concentration of AGEs in plasma proteins and skin collagen in diabetics was associated with higher risk of vascular complications (Ahmed *et al.* 2005, Hanssen *et al.* 2015) including CAD in diabetes (Hanssen *et al.* 2015, Nin *et al.* 2011).

A study suggests role of MG and AGEs (therefore weaker GLO1) in development of T1D by poor protection of beta cells (Kim *et al.* 2013). Another studies point towards dicarbonyl stress as promoter of insulin resistance leading to T2D (Maessen 2014, Nigro *et al.* 2014).

Together with the fact that GLO1 activity is decreased in hyperglycemia these observations and knowledge about *GLO1* regulation have led to idea to treat diabetes with *GLO1* expression inducers activating Nrf2 (Xue *et al.* 2012). One such activator currently is in phase 2 clinical trial to assess its potential in treating diabetic nephropathy as well as other microvascular complications of diabetes and cardiovascular disease (Xue *et al.* 2016).

1.3.3. Genetics of glyoxalase system in diabetes and other conditions

Gene encoding GLO1 is located in human 6th chromosome at p21.2 locus, spans 27.22kb and has only two transcripts. Other member of glyoxalase system *HAGH* (hydroxyacylglutathione hydrolase) is located at 16p13.3 and spans 31.57 kb on the reverses strand of DNA (Yates *et al.* 2016).

Genomic polymorphisms in *HAGH* are rare. Six SNP with global minor allele frequency (MAF) above 1% are known (as of April 2017). Five of them are synonymous and one missense without known phenotype alteration. Only two are present in population with European descent where also population of Latvia belongs (Yates *et al.* 2016).

Two relatively common (global MAF above 20%) *GLO1* SNP are known and present in human populations worldwide. There are also studies published about these associating *GLO1* SNP with increased prevalence of diabetic neuropathy (Groener *et al.* 2013), cardiovascular complications (Rabbani and Thornalley 2011) and autism (Barua *et al.* 2011). The most studied is adenosine to cytosine exchange in base 419 in exon 4 of *GLO1* (rs4746 also known as rs2736654). It is missense SNP where Ala111 is substituted with glutamic acid. Proteomic study discovered that GLO1 with glutamic acid has lower activity and higher acidity than wild type protein (Junaid *et al.* 2004). This was confirmed in a study by Barua *et al*. who found reduced activity of enzyme, elevation of MG and RAGE in patients with autism (Barua *et al.* 2011) and a study which found significantly higher rate of cardiovascular disease and peripheral vascular disease in patients requiring hemodialysis (Kalousova *et al.* 2008) .

Meanwhile, Dutch study found no association of rs4746 and other eight *GLO1* polymorphisms with vascular complications in T2D patients (Engelen *et al.* 2009).

It was found that rs4746 allele frequency is not in Hardy-Weinberg equilibrium in patients with Alzheimer's disease, but no significant association was found (Chen *et al.* 2004). Investigation into whether rs4746 carriers have increased enzyme activity in T1D patients with normal renal activity lead to conclusions that there is no significant association as well as no association with serum glucose, glycated hemoglobin and AGEs levels (Sakhi *et al.* 2013).

Literature is sparse about other *GLO1* polymorphisms. SNP rs1049346 in *GLO1* promoter (but not rs4746) was associated with diabetic retinopathy and nephropathy in Chinese T2D patients (Wu *et al.* 2011).

It is known that *GLO1* is located in region with copy number variation in approximately 2% of humans. This causes 2-4-fold increase in *GLO1* expression, but there is no study published with assessment if these individuals are more protected from formation of AGEs and/or diabetic complications (Cahan *et al.* 2009, Redon *et al.* 2006).

1.4 Pituitary adenomas

1.4.1. Pituitary location, anatomy and functions, embryonal development

Pituitary is an endocrine gland located in human head at the base of brain. Superior to sphenoid bone in a cavity called sella turcica and below chiasma and hypothalamus. Pituitary is connected to hypothalamus via pituitary stalk through which blood supply is organized and control from hypothalamus is executed. Pituitary consists of three lobes adenohypophysis (pars anterior, anterior lobe), neurohyphophysis (pars posterior, posterior lobe) and pars intermedia (intermediate lobe) which is rudimentary in adult humans. Each of the lobes has separate development and functions (Graaff 2001).

Organogenesis of pituitary begins at 4th week of embryogenesis and can be described as three stage process. First initiation of organogenesis and Ratke's pouch occurs, after which evagination of Rathke's pouch and cell proliferation follows and the last stage of pituitary organogenesis is cell lineage determination and cellular differentiation. Briefly at closer look pituitary develops from fusion of two types of ectoderm. Adenohypophysis starts from a thickening of cells in the oral ectoderm (which is of epithelial origin and forms early roof of the mouth) and formation of hypophyseal placode which grows towards neural ectoderm to become Rathke's pouch. At the same time ventral diencephalon (ectoderm of neural origin) forms extension growing downwards toward Rathke's pouch, connecting with it and forming pituitary. Rathke's pouch separates from oral ectoderm at week six to eight. Then cells of anterior wall of Rathke's pouch proliferates and develops adenohypophysis, posterior wall divides slowly and give rise to pars intermedia. Cells from ventral diencephalon form neurohyphophysis. Final patterning and differentiation of adenohypophysis cells forms the five main specialized cell types of the pituitary. Given the nature of pituitary organogenesis, precise coordination and regulation at both locations is crucial for formation of pituitary and correct differentiation of hormone-producing cell types (Larkin and Ansorge 2000) Mutations in key developmental genes can lead to various developmental disorders (Davis *et al.* 2010, Kelberman *et al.* 2009, Zhu *et al.* 2007).

Hormones produced and secreted by adenohypophysis are: human growth hormone (GH or growth hormone), regulated by growth hormone-releasing hormone and somatostatin, thyroid-stimulating hormone (TSH) regulated by thyrotropin-releasing hormone and somatostatin, luteinizing hormone and follicle-stimulating hormone regulated by gonadotropin-releasing hormone, corticotropins – peptide hormones (adrenocorticotropic

hormone (ACTH), melanocyte stimulating hormone and β-endorphin) produced after processing precursor proopiomelanocortin and are regulated by corticotropin-releasing hormone and prolactin (PRL) which is released after signal from several factors (including thyrotropin-releasing hormone, oxytocin, vasopressin, vasoactive intestinal peptide, angiotensin II, neuropeptide Y, galanin and neurotensin) depending on situation. PRL release is downregulated by dopamine. Adenohypophysis is regulated by hypothalamus via previously mentioned release hormones that are secreted in adenohypophysis and delivered there by capillaries forming hypothalamic-hypophysial portal system (Institute for Quality and Efficiency in Health Care 2015).

Intermediate lobe produces and secretes melanocytes stimulating hormone. Posterior lobe of pituitary does not produce hormones, but stores and releases hormones (antidiuretic hormone (vasopressin) and oxytocin) produced by magnocellular neurosecretory cells of hypothalamus into the bloodstream (Institute for Quality and Efficiency in Health Care 2015).

1.4.2. Incidence of pituitary adenomas

PA are neoplasms (new and abnormal growth of tissues) of the pituitary with benign characteristics. Up to 15% of clinically active intracranial neoplasms are PA (Jagannathan *et al.* 2005). Prevalence of PA are high with different samples showing range from 1.5% up to 27% in general population (Molitch 2009) with meta-analysis estimation of prevalence standing at 16.7% (Ezzat *et al.* 2004). Clinically significant adenomas are much rarer ocasion one in 1000 to 1300 individuals (Daly *et al.* 2006, Fernandez *et al.* 2010) although there are reports of prevalence up to one PA in 200 individuals (Rosario 2011). Incidence of PA stands between 0.22 to 8.2 new PA cases per 100,000 per year ((Monson 2000) after Gold 1981). PA is age related with significant increase of cases and incidence after age of 45 ((Monson 2000) after Gold 1981). PA is associated with female sex, epidemiological data show that approximately 2/3 of PA occur in females (Gruppetta *et al.* 2013, Monson 2000) (prevalence males 46/100,000 and females 105/100,000 (Gruppetta *et al.* 2013).

1.4.3. Types of pituitary adenoma

PA are usually classified either by size, extension or function according to the aim of the classification. Hardy classification use four grades to describe PA by their size <10mm (grade I), >10mm within sella (grade II), >10mm outside sella (grade III) and large adenomas with invasion in surrounding tissue, including but not limited bone, brain, compression of optical

nerve (grade IV) (Hardy 1969). Also PA are more informally categorised by their size into two groups: microadenomas (less than 10mm in diameter) and macroadenomas (more than 10mm). This classification seems to originate from requirements of surgery and origin is lost in time.

Classification by extension focuses on invasive PA which is described in details at Micko et al and in brief, uses intracavernous and supracavernous internal carotid arteries as a reference (Micko *et al.* 2015).

Functional classification uses disruptions in patients hormonal profile to classify PA. Between 35-40% of PA are non-functional (NFPA, does not secrete hormones, to cause detectable hormonal change) and other 60-65% are hormones secreting PA. Most common functional adenoamas are prolactinomas (accounting for around 40% of all PA), followed by growth hormone (GH) secreting PA (~13%)(often leading to acromegaly), ACTH secreting PA (~8,5%)(causing Kushing's disease) and TSH secreting and others, including mixed PA (~1%)(Famini *et al.* 2011). Table 4. Also morphological classification is used for some secretory types of adenomas (Syro *et al.* 2015), especially detailed it is developed in somatotropinomas leading to acromegaly.(Cuevas-Ramos *et al.* 2015).

Upcoming PA classification by World Health Organisation is set to be published in 2017 based on subclassification with pituitary transcription factors (Shaid and Korbonits 2017).

Table 4. Frequency of PA types among all patients, sex and ages. (Famini *et al.* 2011).

Diagnose	Total N	N		Age range (average)	
		Males	Females	Males	Females
Prolactinoma	395	90	305	17-81 (46)	15-89 (36)
NFPA	364	162	202	18-85 (54)	17-87 (47)
GH adenoma	127	59	68	19-84 (49)	18-82 (45)
ACTH adenoma	84	18	66	19-77 (43)	10-66 (38)
GH/PRL mixed	4	3	1	24-42 (32)	15
Nelson's syndrome	2	0	2	-	44-66 (50)
Pituitary carcinoma	2	2	0	45-75 (60)	-
Luteinizing hormone/follicle-stimulating hormone adenoma	1	1	0	59	-
GH/TSH mixed adenoma	1	0	1	-	31
TSH adenoma	1	0	1	-	55

N – number of patients, NFPA - non-functional pituitary adenoma, GH – growth hormone, ACTH - adrenocorticotropic hormone, PRL - prolactin, TSH - thyroid-stimulating hormone.

1.4.4. Genetics of pituitary adenomas

Both genetic and epigenetic factors are involved in development of PA (Peltomaki 2012). Yet majority of PA genetic background remains undiscovered. PA are thought to be of monoclonal origin (Jacoby *et al.* 1990). Current literature suggest that up to 95% of PA are

sporadic where somatic mutations, mosaic mutations, and familial low penetrance mutations are the cause of neoplasm (Shaid and Korbonits 2017). Another studies show that clinically significant adenomas tend to occur in families (Couldwell and Cannon-Albright 2010) and syndromic disease, positive family history, childhood-onset adenomas are the most important risk factors for prediction of PA (Shaid and Korbonits 2017).

1.4.5. Genetics of sporadic pituitary adenoma

Sporadic PA account for about 95% of pa cases. Over time mutations in several genes have been found to contribute towards PA in specific or broader range of cases. Heterogeneity of causes is the reason why PA can be considered fully multifactorial disease (Zhou *et al.* 2014).
GNAS is located at chr20 q13.32 locus on the forward strand. It spans 71,47kb and at least 58 transcripts have been detected (Yates *et al.* 2016). This gene encodes G protein α subunit and mutation can nullify or greatly diminish GTP-ase activity of the α subunit and it becomes constitutively active (permanently switched on). Then, through increased production of cyclic adenosine monophosphate and its dependent PKA, which ensures binding of CREB to the PIT1 promoter regions, GH synthesis increases and cell proliferation follows (Shaid and Korbonits 2017). Two common *GNAS* mutations are p.R201C/S/H and p.Q227L/R (Freda *et al.* 2007, Landis *et al.* 1989, Patten *et al.* 1990). Former accounting for majority of cases – 89% (N=8) (Taboada *et al.* 2009) and 95% (N=21)(Shi *et al.* 1998) while latter was found in one case in each of these studies. *GNAS* mutation p.R201C has been reported in one corticotropinoma (Riminucci *et al.* 2002) and one NFPA (Taboada *et al.* 2009).
Some studies report that 40% of somatotropinomas harbor somatic mutations in *GNAS* (Freda et al. 2007, Landis et al. 1989). Other studies in different population put this number at around 15% (Ronchi *et al.* 2016, Taboada *et al.* 2009).
USP8 (ubiquitin specific peptidase 8) is located at 15q21.2 on forward strand spanning 90.04kb (Yates *et al.* 2016). Nineteen transcripts encode at least seven proteins ranging from 62 to 1118 amino acids. *USP8* encodes enzyme ubiquitin carboxyl-terminal hydrolase 8 which removes ubiquitin from other proteins, yet is also capable of promoting receptor degradation and is involved in protein "sorting machinery" within a cell (Berlin *et al.* 2010). Mutations in *USP8* were identified in four out of ten corticotroph adenomas during exome sequencing. Mutations increase activity of the enzyme and increased amounts of various proteins, especially epithelial growth factor (EGF) receptor (Reincke *et al.* 2015). Increased EGF signaling leads to increased cell proliferation (Normanno *et al.* 2006). Initial discovery

that *USP8* mutations cause corticotroph PA has been replicated at least twice (Ma *et al.* 2015, Perez-Rivas *et al.* 2015)and in one of replications involve East Asian population (Ma *et al.* 2015). In the first validation study 134 functioning and 11 silent corticotroph PA were investigated and 48 of functioning PA harbored *USP8* mutations, while none of silent PA did (Perez-Rivas *et al.* 2015). The second study found 67 out of 107 PA to have *USP8* mutations therefore suggesting higher share related to *USP8* somatic mutations (Ma *et al.* 2015). Interestingly PA with *USP8* mutations had higher expressions of pro-opiomelanocortin, SSTR5, and MGMT and were more responsive to the somatostatin analog pasireotide (Hayashi *et al.* 2016). This discovery of *USP8* variations as cause of functional corticotroph PA have helped to identify prime target for drug therapy.

PI3KCA is located at 3q26.32 on forward strand, spans 91.98 kb and encodes at least four transcripts (Yates *et al.* 2016). Gene encodes alpha subunit of PI3K (phosphoinositide 3-kinase), which is integral part of the PI3K signaling pathway and important interacting factor with the AKT and mTOR pathway (Robbins and Hague 2015). It has been described as oncogene for a long time and is the most frequent oncogenic pathway in breast cancer and in ~30% of cases *PI3KCA* mutations are found (Mukohara 2015). The first study to investigate *PI3KCA* mutations in PA were published in 2009 and it was found that 8/91 invasive PA (8,8%) had *PI3KCA* mutations, while none of 262 noninvasive PA did. Similar to cancers, mutations were found in exons 9 and 20 of the *PI3KCA* gene (Lin *et al.* 2009). Another study in Brazil investigated PI3KCA mutations in 33 PA. previously reported mutations were found in 20th exon. Three tumors were NFPA and invasive while fourth was corticotroph microadenoma (Murat *et al.* 2012). Although size of studies is not directly comparable, results indicate that *PI3KCA* mutations might contribute to development of PA.

Recently, several studies using whole exome and genome sequencing have been performed to search for novel PA causing mutations in genomic and tumor DNA pairs. Newey *et al*. in 2013 sequenced exome of seven PA and blood derived DNA pairs. They found low levels of somatic mutations, which relates to benign nature of PA. The average mutation rate was 3.5 per adenoma (range 1-7) and no recurrent mutated genes were found even with 24 PA follow-up group (Newey *et al.* 2013). Finnish study focused on GH secreting PA. Analysis of twelve adenoma and corresponding blood DNA revealed on average 129 somatic mutations per genome and 2.3 single nucleotide variation per exome. Only recurrent mutations were previously reported *GNAS* (at codon 201) in three cases. Several chromosome loses (in chromosomes 1, 6, 13, 14, 15, 16, 18, 22) were detected and one patient had complex

chromosome rearrangement. Five tumors had somatic mutations in genes of the Ca2+ and ATP pathways (Valimaki *et al.* 2015). A study of east Asian population took systematic approach of sequencing seven types of PA: 20 NFPA, 20 PRL secreting PA, 20 GH secreting PA, 20 ACTH secreting PA, 20 gonadotropins (including follicle stimulating hormone and luteinizing hormone) secreting PA, 10 TSH secreting PA, and 15 mixed PA secreting at least two hormones. Besides *USP8* (in ACTH secreting PA), *GNAS* (in GH secreting and mixed secreting GH and other hormone) and *MEN1* (in mixed PA), six other genes showed somatic mutations in at least two tumors (*KIF5A*, *GRB10*, *NR3C1*, *TRIP12*, *SP100*, *IARS*) providing insight into potential drivers of PA development in some cases. Authors also report median somatic mutation rate per adenoma exome which is 3.3 (range 0-13) (Song *et al.* 2016). Also a study identified heterozygous missense mutation c.4136G>T (p. Arg1379Leu) in cadherin-related 23 (*CDH23*) in a family with inherited PA, gene was later screened in familial PA, sporadic PA and healthy controls. Results revealed that 4/12 families (33%) and 15/125 (12%) sporadic PA patients had variants in *CDH23* providing yet another candidate to understand PA genetics (Zhang *et al.* 2017).

1.4.6. Genetics of familial pituitary adenoma

Familial PA are described as rare and encompass about 5% of all clinically significant PA (Shaid and Korbonits 2017). Multiple endocrine neoplasia (MEN) type 1 is an autosomal dominant disease caused by mutation/s in *MEN1* gene. Approximately 20-50% MEN cases develop PA and also adenomas in other endocrine glands (Schernthaner-Reiter *et al.* 2016). *MEN1* is located at 11q13.1 on reverse strand and spanning about 7.79kb. Ten exons give rise to at least 16 transcripts of which 14 have been found as proteins (Yates *et al.* 2016). Most transcripts encode either 610 or 615 amino acid protein MENIN which is tumor suppressor which regulates promoter activity of genes controlling cell cycle and proliferation via interaction with histone methyltransferase complex. Which in turn changes epigenetic modification of histones by transfer of methyl groups to lysine and arginine residues (Hughes *et al.* 2004) PA caused by MEN can be described according to Knudson two hit hypothesis of tumorigenesis (Knudson 1993). The first mutation is inherited and the second is somatic often resulting in loss of heterozygosity of the *MEN1* locus or disrupting *MEN1* microRNA mediated expression. Almost half of cases have large deletion as a second hit, 25% have nonsense mutation, 15% insertion, 10% have missense mutation and few percent have splice site mutations (Lemos and Thakker 2008). Patients with PA caused by MEN usually have

more aggressive tumors, with larger size and more pronounced symptoms (Syro *et al.* 2012, Verges *et al.* 2002).

Next to the *MEN1* at locus 11q13.2 on the same reverse strand is located aryl hydrocarbon receptor interacting protein (*AIP*) gene (Yates *et al.* 2016). Heterozygous mutations in AIP cause autosomal dominant disorder familial isolated pituitary adenoma (FIPA) with ~20% - 60% penetrance (Igreja *et al.* 2010). This is an early onset disorder (before age of 30) and mostly presenting as GH secreting adenoma with increased invasiveness and reduced response to drug treatment (Shaid and Korbonits 2017, Vierimaa *et al.* 2006). Mutations that cause only partial protein product are the most common in *AIP* related FIPA. *AIP* mutations have been found only in 20% of FIPA patients (Leontiou *et al.* 2008) and other known gene associated with FIPA is duplicated *GPR101* (Beckers *et al.* 2015). Rest of FIPA cases have unknown genetic cause. *AIP* R304Stop mutation have been found in Italy (Ferrau *et al.* 2016), United Kingdom (Chahal *et al.* 2011) and Mexico (Ramirez-Renteria *et al.* 2016). Expression of *AIP* has been shown to be better marker of invasiveness than widely used MKI67 (Kasuki Jomori de Pinho *et al.* 2011) and predicts response to octreotide therapy independent of *SSTR2* expression (Kasuki *et al.* 2012).

MEN type 4 is a MEN like disorder but without mutations in *MEN1*. About 33% of MEN type 4 patients develop PA of all types. Using information from rat studies causal gene was identified *CDKN1B* (Pellegata *et al.* 2006). But it is not found in all patients (Thakker 2014). Mutations in *CDKN1B* leads to disrupted cell cycle arrest (Moller 2000).

Carney complex (CNC) can be described as a type of MEN (Stratakis and Ball 2000). Around 10% of patients have PA secreting either GH or PRL. The causative gene has been identified as *PRKAR1A* (by two research group independently (Casey *et al.* 1998, Casey *et al.* 2000, Kirschner *et al.* 2000)), which in case of CNC functions as classic tumor suppressor - mutations are associated with loss of wild-type allele in CNC tumors (loss of heterozygosity occurs) (Kirschner *et al.* 2000). Around 70% of CNC cases are familial, with rest being sporadic. Penetrance of *PRKAR1A* cause CNC is close to 100% (Stratakis *et al.* 2001). Early in the research of CNC linkage studies suggested 2p16 locus as the cause of CNC, but no causative gene has yet (as of 2017) been identified in this region (Stratakis *et al.* 1996).

Xq26.3 duplication acrogigantism (X-LAG) was discovered in a study of early childhood gigantism. Thirteen patients were identified with microduplication and ten of them were carriers of pituitary macroadenoma with median maximum diameter 16mm. Other three had pituitary hyperplasia. Drug therapy was not sufficient in any of patients to achieve

hormonal control. The duplicated genomic segment on X chromosome was about 500 kb long, starting at 135,627,637 and spanning to 136,118,269 (GRCh37/hg19). Yet each patient had unique start and end positions of the duplication, but smallest regions of overlap for all patients were two and encompassed four genes *CD40LG*, *ARHGEF6*, *RBMX* and *GPR101* (Trivellin *et al.* 2014). Functional investigation of these for genes revealed that *GPR101* was expressed up to 1000 times more in pituitaries of Xq26.3 duplication carriers compared to non-carriers. Other three genes were either normally expressed or not expressed at pituitary at all. Same study also tested 248 patients with sporadic PA and no Xq26.3 duplication was found, but 11 patients had a c.924G→C (p.E308D) substitution in *GPR101*, which was not found in 7600 controls (Trivellin *et al.* 2014). *GPR101* is coding orphan G-protein–coupled receptor with high expression in rodent hypothalamus (Bates *et al.* 2006). Previously p.A397K mutation has been described and patented as constitutive activator of receptor in mice (Lowell 2011). Mutation p.E308D is physically close to p.A397K in GPR101 model. Overexpression of p.E308D and p.A397K GPR101 mutants in rat GH3 cells, but not wild type receptor lead to significant increase of cell proliferation and secretion of growth hormone (Trivellin *et al.* 2014).

1.4.7. Somatostatin system

Somatostatin (SST, also known as growth hormone–inhibiting hormone or growth hormone release–inhibiting hormone or somatotropin release–inhibiting factor somatotropin release–inhibiting hormone) is a peptide hormone regulating endocrine system (Brazeau *et al.* 1973) and influencin neurotransmission (Epelbaum *et al.* 1994, Patel *et al.* 1995) and cell proliferation (Stewart and James 1999) via interaction of five somatostatin receptors which belong to G-protein coupled receptor family (Gahete *et al.* 2010). Somatostatin inhibits secretion of insulin, glucagon (Krantic 2000) and growth hormone (Brazeau *et al.* 1973, Panetta *et al.* 1994). There are five somatostatin receptors discovered in humans to date (SSTR1, SSTR2, SSTR3, SSTR4 and SSTR5 (Reisine and Bell 1995)) and also three natural antagonists are known cortystatin (Dalm *et al.* 2003, de Lecea *et al.* 1996) and two types of SST – somatostatin-14 and somatin-28 (Krantic 2000).

Human genes for somatostatin receptors *SSTR1* (located at chr14q13), *SSTR2* (17q24), *SSTR3* (22q13.1), *SSTR4* (20p11.2) un *SSTR5* (16p13.3) do not contain introns (Yates *et al.* 2016, Krantic 2000). Functional receptors of all five types have been identified in central nervous system and endocrine glands. *SSTR1* and *SSTR2* are widely expressed throughout the body in

various tissues, while *SSTR3*, *SSTR4* un *SSTR5* expression is more localised. Meanwhile *SSTR4* mRNA have not been found in human pituitary (Dalm *et al.* 2003).

SSTRs inhibit adenocyclasis (Gα 1-3) while selectively activating other transducers, such as phospholipase-Cβ as well as activating internally regulating K^+ ion channel and current dependent Ca^{2+} ion channel (Patel 1999).

The hypothesis stands that specific response to SSTR activation is dependent on co-expression of SSTRs within the cell and interaction between functioning types of SSTR and their quantity (Cakir *et al.* 2010). SSTRs also can be constitutive activated (Ben-Shlomo *et al.* 2013) and are regulated by endocytosis, internal trafficking and arrestin-mediated desensitization (Hofland and Lamberts 2003, Tulipano *et al.* 2004).

1.4.8. Natural ligands of somatostatin receptors

There are two naturally active forms of somatostatin (somatostatin-14 and somatin-28) and they have similar affinity to SSTR1-4 and in case of somatostatin-14 also to SSTR5, but somatostatin-28 has higher affinity to SSTR5 (Bruns *et al.* 2002).

Cortystatin (CST) is cyclic neuropeptide consisting of eleven amino-acids which are similar to those of somatostatin (de Lecea *et al.* 1996). CST has affinity to all five SSTRs and is influencing neuronal activity and cell proliferation. CST is also involved in sleep pattern regulation and reduction of locomotor activity (Spier and de Lecea 2000) CST not only binds to SSTRs but also GH receptor (Deghenghi *et al.* 2001). CST and SST have similar effect on secretion of GH, PRL and insulin in patients with acromegaly or prolactinoma (Grottoli *et al.* 2006).

1.4.9. SSTR5 and pituitary adenomas

Somatotatin receptor 5 (SSTR5) was discovered in 1993 (Sandford *et al.* 1996). *SSTR5* is expressed in human brain and also in pituitary in adults and both pituitary and hypothalamus during human embryonic development (Patel 1999). There are two isoforms of mature SSTR5 consisting of five or four transmembrane domains and these isoforms are called SST5TDM5 and SST5TDM4, respectively. Both isoforms are functionally active but with different ligand binding properties. SST5TDM5 is preferably binding somatostatin (60%) and not more so CST (12%), meanwhile SST5TDM4 binds CST (43%) and only minute quantities of somatostatin (4%). Expression is differentiated between the two isoforms as well. SST5TDM5 is expressed in tissues throughout the body and in a study was detected in seven out of 34

NFPA. SST5TDM4 isoform was rarely detected in normal tissues but found in 95% of PAs including somatotropinomas, corticotropinomas, NFPAs (Duran-Prado *et al.* 2009). A study in 2000 discovered that SSTR5 together with dopamine D2 receptor (DRD2) is involved in formation of hetero-oligomers. This complex has elevated functional activity and leads to synergic reactions. Complexes with DRD2 could mean that SSTR5 in involved in psychiatric disorders and also suggest a role of DRD2 in pituitary (Rocheville *et al.* 2000). Clinical importance of SSTR5 is huge in case of GH secreting PAs. SSTR5 inhibits secretion of GH in somatotropinomas, leading to successful drug use in therapy of acromegaly and GH oversecretion (Saveanu *et al.* 2001).

Literature mentions two polymorphisms that are clinically relevant: T461C and C1004T are associated with level of GH and IGF-1 in patients with acromegaly (Filopanti *et al.* 2005).

2. Materials and Methods

2.1 LGDB DNA samples and phenotypic information

All four studies were performed using genomic DNA samples acquired from the Latvian Genome Database (LGDB) biobank, which is funded by the Ministry of Health, Republic of Latvia. Participants are recruited and health status recorded by medical personal in hospitals and general practitioners located in Latvia. Individuals have to be at least 18 years old. Diagnoses are based on approved criteria according to International Classification of Diseases (ICD-10) codes. Anthropometric measurements (including weight and height) are acquired during a questionnaire-based interview. Ethnic, social, lifestyle and environmental information, as well as familial health status are self-reported and isn't crosschecked. Recruitment was population based; a specific health condition was not required for involvement. The studies were carried out in accordance with the Declaration of Helsinki. The protocols for each individual study was approved by Central Medical Ethics Committee of Latvia. Written informed consent was obtained from all participants in all studies.

DNA is extracted from the whole-blood leukocytes by phenol–chloroform method.

DNA samples for the studies were provided by LGDB and aliquoted into 96 well PCR plates or PCR tubes by Tecan Freedom Evo robotic pipette (Tecan, Männedorf, Switzerland). Final DNA amount was 28ng per well.

2.2 Study group description criteria

We followed the Strengthening the Reporting of Genetic Association guidelines to describe selection of the study group and association analyses (Little *et al.* 2009).

2.3 Study population

2.3.1. Coronary artery disease study

Study groups were selected from all 7936 participants recruited for the LGDB project from 2003 to 2008. We initially excluded patients with cancer, renal diseases, variant angina (ICD-10 I20.1), cardiomyopathy (ICD-10 I42 and I43), and rare diseases (a complete list is available upon request). Participants with missing phenotypic data analyzed in this study were also excluded. After these exclusions were carried out, patients with CAD manifestations including angina (ICD-10: I20) and MI (ICD-10: I21 and I25.2) were selected

for the case group. The final number of patients was 685. To avoid selection bias based on the hospital or a specific disease group, controls were randomly selected only from LGDB participants recruited by general practitioners. Patients with type-2 diabetes (ICD-10: E11) were not excluded from either case or control group. A total of 830 controls without cancer, CAD and its manifestations, renal or hepatic conditions, or thyroid disease were used in the present study. Information about smoking status, alcohol intake, and physical activity were self-reported. Individuals were designated ‘‘smokers’’ if they have smoked ≥ 5 cigarettes a day for ≥ 12 months. Former smokers and current smokers were classified according to these criteria. Physical activity was classified according to self-reporting of previous physical activity. The non-consumers group comprised individuals who had the following behaviors: not consuming alcohol; consuming alcohol less than once a week; or drinking less than 1.5 units of alcohol per week. The remainder of the group was designated ‘‘alcohol consumers.’’ The study was approved by the Central Medical Ethics Committee of Latvia (Resolution A-5, March 22, 2007).

2.3.2. GLO1 activity study

In total, 326 participants were recruited for case-control study: 101 patients with T1D, 100 patients with type 2 diabetes T2D, and 125 non-diabetic individuals. All control participants and patients were of European descent. Exclusion criteria were chronic renal failure (glomerular filtration rate less than 60 ml/min), chronic liver disease, an active inflammatory disorder (white blood cell count > 8.5 × 103/mm3), an erythrocyte sedimentation rate > 20 mm/h, or high-sensitivity C-reactive protein > 15 mg/l (because high levels may indicate a latent chronic inflammatory condition), anemia, current evidence or a history of malignancy in the last 5 years, a history of drug or alcohol abuse, and causes of neuropathy other than diabetes. The study was approved by the Central Medical Ethics Committee of Latvia (Resolution A-9, May 29, 2006).

2.3.3. Pituitary Adenoma study 2009

The acromegaly patients (total N=48) used in this study were enrolled to the LGDB from 2004 and 2008 from two main hospitals, Pauls Stradins Clinical University Hospital (N=41) and Riga Eastern Clinical University Hospital (N=7) and they represented approximately 80% of all the acromegaly patients registered in Latvia as of October 2008 (diagnosed with acromegaly from 1985 to 2007). Additional data were collected based on hospital

records and interviews for all the patients selected for this study (ICD-10 code E22.0). 45 patients received the somatostatin analogs octreotide (Sandostatin®LAR) at dose 10-30 mg in every 28 days and lanreotide (Somatuline®Autogel®) at dose 60-90 mg every 28 days. Tumor size was measured as the maximum diameter obtained from magnetic resonance imaging data and tumors were classified accordingly as microadenomas (<10mm) or macroadenomas (>=10mm). The effect of somatostatin analogs on tumor proliferation and insulin-like growth factor 1 (IGF-I) level normalization was estimated by comparing the tumor sizes and serum IGF-I (μk/ml) measurements during the course of therapy. In order to estimate the dynamics of adenoma size, the data from last follow-up was compared with the first available measurement with at least 12-month period in between. We excluded all cases where the therapy was interrupted or adenoma resections were performed during this period of time. Two groups were defined: "reduced" with observable tumor shrinkage (N=11) and "unchanged" with no observable tumor shrinkage (N=22) together with the cases that showed prolonged expansion (N=2). Only a limited number of cases had IGF-I measurements available before the therapy. For the IGF-I response, only the data at least 6 months after the start of the SA therapy were considered for analysis and only if the therapy was not interrupted. In those cases, where several IGF-I measurements were available, the mean IFG-I was calculated, excluding the outliers when that was possible. Non-responsiveness was defined as the mean IFG-I value above the upper limit of the normal value at the corresponding age. Due to the lack of uniformity in the GH measurements, we did not include the GH levels in this analysis.

Two independent control samples were used in this study in order to minimize the risk of false association.

Control I was 96 samples randomly chosen for sequencing from total of 2203 LGDB participants recruited from 2003 to 2005 excluding patients with metabolic and endocrine diseases. Control II group was sex and age matched to cases and consisted of 475 LGDB participants. This group was selected from all of the 7935 LGDB participants recruited from 2003 to 2008 applying the following selection criteria. First we excluded subjects having any chronic disease including metabolic, endocrine, coronary heart, cancer and renal conditions. Similarly, we excluded all participants with missing the relevant phenotypic data. After this selection 1225 healthy adults were considered for further use. Matching by age was performed in ten-year age groups randomly choosing ten sex matched controls for each acromegaly patient from the corresponding age group. Five

samples were omitted due to the practical reasons (96 plate format). Under these criteria 63 participants from the Control I were also included in the Control II group. Study protocol was approved by Central Medical Ethics Committee of Latvia (Protocols No A-33, 2005 and A-3, 2008).

2.3.4. Pituitary adenoma study 2016

The case group (165 participants) was enrolled for this study from the LGDB between 2004 and November 2011. Two university hospitals involved in the treatment of PA in Latvia participated in the enrollment of study cases. One hundred and fifty-eight patients were recruited from Pauls Stradins Clinical University Hospital and seven patients were from Riga Eastern Clinical University Hospital. Hormonal profiles (typically levels of GH, prolactin, IGF-I, ACTH, and others, depending on the presence of clinical indications) from the blood were determined at an independent commercial laboratory (E. Gulbis Laboratory, Riga, Latvia) and used for the diagnosis. Prolactin was detected by electrochemical luminescence with Cobas 6000 (Roche, Basel, Switzerland), while GH, IGF-I, and ACTH levels were measured with Imulite 2000 (Siemens AG, Berlin, Germany). In cases with elevated levels of these hormones, magnetic resonance imaging (MRI) was performed. The presence of a pituitary tumor was determined using high-definition MRI data from a 1 mm slice series of the pituitary gland without contrasting agent with a Siemens Magneton 1.5T (Siemens AG, Berlin, Germany). During the first visit to the endocrinologist, phenotypic features (primary diabetes, primary hypertension, heart dysfunction, carpal bone syndrome, sleep apnea, macrognathia, and morphology of the nose and frontal bone) were also considered in the diagnosis, and further blood serum hormone testing was performed. Data used in this study include sex, age, age at tumor diagnosis, PA size, presence of extrasellar growth, and hormone-secreting type, treatment type, drug prescription information, and the occurrence of other tumors. PA was designated as a “macroadenoma” if its diameter according to the MRI data was larger than 10 mm. It must be noted that age at diagnosis does not correspond to the age of tumor incidence or age when clinical symptoms attributed to PA occurred. Phenotypic data were collected based on hospital records and interviews for all the patients selected for the study. Samples were excluded from the study due to missing phenotype data (age or sex; N=9), misdiagnosed as having PA in the primary examination (N=5), inadequate DNA sample quality (N=2), or re-diagnosis as a pituitary carcinoma (N=1). Due to the low

number of cases, we also excluded patients with Cushing's disease (N 4). This left 144 cases eligible for genotyping. Age- and sex-matched controls were selected from LGDB participants without endocrine or metabolic diseases or other chronic disease. Matching of samples was performed by dividing the case sample into groups based on sex, then further dividing subjects into age bins by decade of age. The sample of suitable controls (N=738) was divided in a similar manner. Controls were picked randomly from each subgroup according to a number based on each respective case group. Samples with phenotypic information missing (e.g., sex, age, or BMI) were excluded from random matching. It should be noted that sex and age matching was performed before genotyping, and thus, it was influenced by exclusion of some samples as a result of genotyping quality check. However, the medians of age and interquartile range remained similar for all respective case–control groups, and in case of linear and logistic regression, sex was used as a covariate controlling for this issue. A total of 365 controls and one inter-plate positive control were used in this study. The case-to-control ratio was 1:2.5. The study protocol was approved by the Central Medical Ethics Committee of Latvia (protocol no. 01.29.1/28, December 14 2011).

2.4 Target selection of genetic variants

2.4.1. Coronary artery disease study

The aim of the study was to evaluate the role of non- synonymous SNPs in ADORA3 with respect to susceptibility to CHD. Four nonsynonymous SNPs have been detected in ADORA3 and are listed in the SNP database of the National Center for Biotechnology Information (dbSNP, build 131): rs55653224, rs35986308, rs35511654, and rs2800889. We considered only SNPs that were present in a population of European descent with a minor allele frequency (MAF) of ≥0.01. Only rs35511654 corresponded to the aforementioned criteria and was therefore selected for analyses.

2.4.2. Glyoxalase 1 study

We selected three SNPs (rs2736654, rs1130534, rs1049346) in the GLO1 gene using the following selection criteria: SNPs located inside the coding region or in close proximity (within 0.1 kb) of GLO1, and SNPs with MAF ≥0.1 in the European population, based on information in the NCBI SNP database (Yates *et al.* 2016).

2.4.3. Pituitary adenoma study 2009

Somatostatin acting through the somatostatin receptors is regulating GH secretion and regulation of tumor proliferation. SSTR2 and SSTR5 are found in high levels in somatotropinomas of pituitary. Also somatostatin analogues octreotide and lanreotide that have the highest affinity to SSTR2 and SSTR5 are used to repress GH synthesis and tumor proliferation in acromegaly patients. Direct sequencing of the SSTR5 gene were chosen to gain comprehensive view of SSTR5 mutations in patients with acromegaly.

2.4.4. Pituitary adenoma study 2016

Seven candidate genes were selected based on an extensive literature search about known and probable PA genetics in February 2011. The genes chosen were AIP, MEN1, GNAS, SSTR2, SSTR5, PRKAR1A, and DRD2. Tag SNPs were selected within these genes and in the upstream and downstream regions in strong linkage disequilibrium ($r2 > 0.8$) with markers within each gene using Haploview v4.2 software (Barrett *et al.* 2005) and HapMap release #28 (NCBI build 36, dbSNP b126, CEU analysis panel) (International HapMap 2003) project information available in February 2011. Nonsynonymous SNPs in these genes were also included, and the remaining two slots in the 96-SNP assay were filled with the most informative tag SNPs from PRKAR1A. Detailed information about the regions chosen and linkage disequilibrium plots are available at supplementary 1. The SNP list generated by Haploview v.4.2 (Broad Institute, Cambridge, USA) was analyzed by the Illumina Assay Design Tool (Illumina, San Diego, USA). SNPs with low predicted genotyping success rates were removed and each gene was reanalyzed in Haploview using the “force exclude” option for the marked polymorphisms. The designated genotyping success rate of 0.5 was deemed acceptable in cases where no other SNP was present to capture the marker or important nonsynonymous variant selected. Otherwise, a “Designability Rank = 1” was required. The final number of SNPs corresponded to the requirements of the Custom VeraCode GoldenGate Genotyping platform, offering discrimination of 96 SNPs in a single well of a 96-well microplate as one of the standard options. A full list of SNPs is located at supplementary 2.

2.5 DNA analysis

2.5.1. PCR, SSTR5 amplification

The SSTR5 gene containing genomic DNA region including 5' and entire coding region (from –2239 to +1294 respective to start codon) were amplified in six PCR reactions. PCR primers for DNA amplification were designed using Primer3 software (primer sequences can be found in Supplementary 3A). Following PCR reaction setup was used: 1mM BD buffer, 2,5mM $MgCl_2$, 0,5 units Hot FirePolymerase, 0,2mM dNTP mix (SolisBioDyne, Tartu, Estonia), 0,3mM primers and 28ng template DNA. PCR temperatures were 95°C – 5min, 40 cycles – 95°C – 30s, 55°C – 30s, 72°C – 1min, and 5min at 72°C for final extension and the PCRs were carried out on Veriti96Therma Cycler (Applied Biosystems, Foster City, USA).

2.5.2. PCR, MEN1 validation

Polymerase chain reaction primers were 5′- CAGAAGGTGCGCATAGTGAG-3′ and 5′- GGTCCGAAGTCCCCAGTAGT-3′. Following PCR reaction setup was used: 1mM BD buffer, 2,5mM $MgCl_2$, 0,5 units Hot FirePolymerase, 0,2mM dNTP mix (SolisBioDyne, Tartu, Estonia), 0,3mM primers and 28ng template DNA. PCR temperatures were 95°C – 5min, 40 cycles – 95°C – 30s, 60°C – 30s, 72°C – 30s, and 7min at 72°C for final extension and the PCRs were carried out on Veriti96Therma Cycler (Applied Biosystems, Foster City, USA).

2.5.3. Agarose gel electrophoresis

The presence of PCR products were confirmed by 0,8% agarose gel electrophoresis.

2.5.4. Purification of PCR products

Dephosphorylation of remaining dNTPs was done with shrimp alkaline phosphatase (Fermentas, Vilnius, Lithuania/ Thermo Fisher Scientific, Waltham, USA) according to manufacturer's protocol. Restriction endonuclease ExoI (Fermentas, Vilnius, Lithuania/ Thermo Fisher Scientific, Waltham, USA) was used to remove leftover primers. This cleanup ensures the best results for Sanger sequencing.

2.5.5. Sanger sequencing

SSTR5 gene amplification products had both strands directly sequenced using a set of 14 primers that were designed on Primer3 Software v.2.2.0 (Universität Heidelberg, Heidelberg, Germany) (Untergasser *et al.* 2012) (Supplementary 3B).

Sanger sequencing was performed to confirm MEN1 rs2959656 heterozygous state in seven carriers discovered by Illumina BeadXpress genotyping. Sequencing primers were 5′- CAGAAGGTGCGCATAGTGAG -3′ and 5′- GGTCCGAAGTCCCCAGTAGT -3′.

Following sequencing reaction setup were used: 1µl BigDye, 2µl 5x BigDye sequencing buffer, 0,5mM corresponding primer, 150-250ng of template DNA (from the previously described PCR reactions) and 5.5µl distilled H_2O. Reaction conditions were set: 96°C – 1min, 25 cycles - 96°C – 10s, 50°C – 5s, and 60°C – 4min. PCR was carried out on Veriti96 Thermal Cycler (Applied Biosystems, Foster City, USA).

The products were purified using Sephadex G50 (Sigma-Aldrich, St. Louis, USA) and sequenced with ABI Prism 3100 (AME Bioscience, Torod, Norway) capillary electrophoresis sequencer. All chromatograms were manually inspected using Contig Express software of Vector NTI Advance 9.0 package (InforMax Inc, North Bethesda, USA) or in case of MEN1 sequencing – FinchTV v.1.4.0 (Perkin Elmer, Waltham, USA). Presence of polymorphisms was also confirmed by opposite strand analysis.

2.5.6. MALDI-TOF genotyping

Genotyping were carried out using mini-sequencing and subsequent MALDI-TOF mass spectrometry analysis. Special primers for mini-sequencing were designed using the program CalcDalton (Kirsten *et al.* 2006)(University of Leipzig, Leipzig, Germany). These primers (Supplementary 4) contained biotin cap on 5′ end and photosensitive linker cleavage site. Mini-sequencing reaction setup: 0,5mM 10xC buffer, 1,25mM $MgCl_2$, 1 unit TermiPolDNAPol (Solis BioDyne, Tartu, Estonia), 0,2mM ddNTPs (Fermentas, Vilnius, Lithuania), 1mM primers mentioned above and 5µl of PCR product.

Mini-sequencing conditions were 95°C – 2min, 99 cycles – 95°C – 10s, 55°C – 10s, 72°C – 10s, and finishing 72°C – 5min. After mini-sequencing products were precipitated on streptavidin coated 384-well plates (Biotez, Berlin, Germany) and subjected for ultraviolet exposure for 15 min. After purification 1µl of DNA mini-sequencing sample where diluted in 1µl of 3 –hydroxypicolinic acid matrix (70mM and 40mM di-ammonium hydrogen citrate), spotted on MTP AnchorChip 400/384TF (Bruker Daltonics, Billerica, USA) and air

dried. MALDI-TOF MS analyses were carried out on MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, USA) using maximum 40 nitrogen laser shots with frequency 16,7 Hz on each sample spot. Oligo calibration standard LMW (Bruker Daltonics, Billerica, USA) were used for mass calibration in interval 1000 - 4000 Da. We used the following mass intervals for the genotyping; rs34037914 intervals 3500 – 4500 Da, for rs169068 – 3000 – 4000 Da, for rs642249 – 4000 – 5000 Da.

Results were analyzed using Genotools SNP Manager software v2.2 (Bruker Daltonics, Billerica, USA) and manually verified.

2.5.7. TaqMan genotyping

DNA samples were aliquoted from storage tubes into 96- well PCR plates using a Tecan Freedom Evo system (Tecan, Mannedorf, Switzerland) with disposable filter tips. DNA concentration was normalized to 7 ng/ml. Genotyping was carried out using an Applied Biosystems TaqMan SNP Genotyping Assay with a modified protocol using 4.75 ml TaqMan Genotyping Mix (Applied Biosystems, Foster City, USA), 0.25 ml SNP genotyping assay ID C_25626674_20 (Applied Biosystems, Foster City, USA) and 5 ml Millipore H_2O (Millipore, Bedford, USA) on a 7500 Real-Time PCR system (Applied Biosystems). AutoCaller 1.1 (Applied Biosystems, Foster City, USA) software was used to simultaneously assign genotype calls for all samples. Samples with unsuccessful genotyping were removed.

2.5.8. Illumina BeadXpress genotyping

All 96 SNPs were genotyped using the Illumina BeadXpress system (Illumina, San Diego, USA) with Illumina GoldenGate genotyping assay (Illumina, San Diego, USA) (Lin *et al.* 2009). Genotyping was carried out according to the manufacturer's instructions. In order to ensure quality control and a high intra-subject concordance rate, 46 or 9.3% DNA samples were randomly chosen and distributed to other plates for repeated genotyping. All quality control steps in the manufacturer's instructions were implemented. One sample was used as a positive control across all plates. There was >99.98% observed genotype concordance; with one mismatched allele call in 46 repeat pairs (one out of 5796 alleles obtained), excluding uncalled alleles. Additionally, one mismatched allele call was observed among seven inter-plate controls; therefore, no assay plates were removed from the study due to excessive genotype mismatch. Primary genotyping data analysis

was performed by Illumina GenomeStudio V2010.3 Genotyping module V1.8.4. software (Illumina, San Diego, USA). The Gene Call threshold was set to 0.25. The cluster images of the signal intensity were manually reviewed.

2.5.9. Glo1 enzyme assay

Glo1 activity was determined in whole blood samples on the day of the blood collection, according to a method described in (Skapare *et al.* 2011).

2.5.10. Statistical analysis

Statistical analyses were undertaken with the PLINK 1.06 and v1.07 software (Purcell *et al.* 2007). Deviation from the Hardy–Weinberg equilibrium was assessed by the exact test described by (Wigginton *et al.* 2005) which is more accurate for rare genotypes. The Cochran–Armitage trend test was used for unadjusted association analysis. Logistic regression assuming an additive, dominant, or recessive mode of inheritance was employed to adjust the analysis for other non-genetic factors. Sex, age, body mass index (BMI), diabetes, smoking, alcohol consumption, and physical activity were included in the logistic regression models as covariates. Linear regression was used to determine relationships between genotypes and quantitative variables with and without covariates (sex, age, BMI, smoking, alcohol consumption, history of statin and ACE inhibitor/ARB therapies).

Haplotype association was performed as implemented in PLINK.

Normal distribution was determined using the Kolmogorov–Smirnov test.

To minimize the false-positive results, given the relatively small sample size, we carried out a permutation test with a 'label swapping' method using identical parameters; 100,000 permutations were used including the correction for multiple testing (EMP2 value).

The Chi-square test and Student's t-test were used to compare characteristics between case and control groups. Mann–Whitney rank sum test was used to compare quantitative variables that are not normally distributed.

P values <0.05 after adjustment for Bonferroni multiple testing correction were considered to indicate statistical significance. P values lower than 0.05 before multiple testing correction were deemed noteworthy and their biological relevance was examined closer.

In the 2009 study of PA the IGF-I data were transformed as a normalized percentage of upper limit of normal (ULN) of appropriate age according to formula (CIGF-I – ULNIGF-I)/ULNIGF-I x 100). Normalized IGF and all other continuous variable displayed normal distribution and were further used in linear regression analysis. Two sided Fisher exact test was used to test the allelic distribution in case of categorical clinical variables, except the analysis of number of adenoma resections where Pearson Chi-Square was calculated from 3x3 table.

Statistical power was calculated using Quanto software v1.2.4 (Natara Software, Naperville, USA) (Gauderman 2002) for SNP with frequencies of 1, 5, 10, and 50% using the assumptions of gene-only hypothesis, log-additive inheritance, and a population risk from 0.001 to 0.01 by 0.05 in a two-tailed test using our sample.

3. Results

3.1 A Nonsynonymous Variant I248L of the Adenosine A3 Receptor Is Associated with Coronary Heart Disease in a Latvian Population

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A Nonsynonymous Variant I248L of the Adenosine A3 Receptor Is Associated with Coronary Heart Disease in a Latvian Population

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Adenosine plays an important part in the cardiac response to ischemia and reperfusion. The human adenosine receptor A3 (A_3R), along with other adenosine receptors, is involved in mediation of those effects. The aim of the study was to ascertain whether the nonsynonymous single-nucleotide polymorphism (SNP) I248L (reference SNP ID: rs35511654) located in the A_3R gene is associated with coronary heart disease (CHD). DNA samples from 683 individuals with CHD and from 826 control subjects selected from the Latvian Genome Database were successfully screened for rs35511654 using the TaqMan SNP Genotyping Assay. We observed a significantly decreased frequency of the rs35511654 C allele in a group of CHD patients compared with that in controls ($p=0.009$). The association remained significant after adjustment for age, sex, and other nongenetic factors ($p=0.02$). These results suggest that A allele of rs35511654 may predispose to CHD.

Introduction

The role of adenosine in cardioprotection against damage induced by ischemia–reperfusion has been intensively studied over the past two decades (Liu *et al.*, 1991; Maldonado *et al.*, 1997; Tracey *et al.*, 1997; Thourani *et al.*, 1999; Xu *et al.*, 2006). The cardioprotective effect of adenosine is mediated via four adenosine receptors (A_1R, $A_{2A}R$, $A_{2B}R$, A_3R) that belong to the G-protein–coupled receptor family (Tucker and Linden, 1993). Adenosine receptors are expressed in a wide range of tissues, including within the cardiovascular system (Su *et al.*, 2004).

The main role of cardioprotection has been allocated to the most-studied adenosine receptor: A_1R (Ely and Berne 1992; Mentzer *et al.*, 1993; Sommerschild and Kirkeboen, 2000; Headrick *et al.*, 2003). $A_{2A}R$ and $A_{2B}R$ are involved in cardioprotection in the heart against ischemic damage as cofactors of an A_1R-mediated pathway (Urmaliya *et al.*, 2009).

The least known of the four adenosine receptors is A_3R. It is thought that A_3R signaling protects cardiomyocytes against damage caused by ischemia via short-term preservation by adenosine triphosphate as well as protection from contractile dysfunction and energy depletion due to normalization of intracellular Ca^{2+}. This protective effect has been observed after induced ischemia in the hearts of mice and rabbits (Liu *et al.*, 1991; Maldonado *et al.*, 1997; Tracey *et al.*, 1997; Thourani *et al.*, 1999; Xu *et al.*, 2006) and administration of doxorubicin in rats (Shneyvays *et al.*, 2001). However, the reasons for the possible action of A_3R in other tissues are controversial. Experiments have shown that administration of an A_3R-selective agonist 20 min before cerebral ischemia extends the area of damaged brain, but that administration of the same agonist 20 min after ischemia results in decreased damage (Von Lubitz *et al.*, 2001). One report stated that A_3R signaling suppresses the release of tumor necrosis factor alpha (TNF-α) from human monocytes (Le Vraux *et al.*, 1993), but a more recent study showed completely opposite results (Zhang *et al.*, 2005). The A_3R receptor is expressed in the heart at a very low level. Hence, either this receptor is very efficiently coupled to the signaling pathways protecting the heart or the cardioprotective effects are also induced by activation of the A_3R in cells outside cardiac tissues (Jacobson and Gao, 2006).

The human A_3R gene (*ADORA3*) is located on chromosome 1p13.2 (HapMap build 37.1) and consists of two exons separated by a 2.2-kb intron (Murrison *et al.*, 1996). One study focusing on the role of adenosine receptors in myocardial infarction (MI) has shown that a nonsynonymous single-nucleotide polymorphism (SNP) rs35511654 located in *ADORA3* is associated with an increased size of the infarct. Single-proton emission computed tomography (SPECT) showed an increase of ~33% in infarct size per C (minor) allele (Tang *et al.*, 2007). No other studies have been published

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on the impact of *ADORA3* variants on coronary heart disease (CHD) and its clinical manifestations.

In the present study, we demonstrated the association of the rs35511654 A allele with CHD in a cohort of patients from the Latvian Genome Database (LGDB).

Materials and Methods

Study population

We followed the Strengthening the Reporting of Genetic Association guidelines to describe selection of the study group and association analyses (Little *et al.*, 2009). Subjects for this case–control study were acquired from the LGDB biobank, which is funded by the Latvian government. Participants were recruited by medical personal in hospitals and general practitioners located in Latvia.

Individuals had to be >18 years of age. Health status was recorded by physicians. Diagnoses were based on approved criteria according to International Classification of Diseases (ICD-10) codes. Anthropometric measurements (including weight and height), ethnic, social, and environmental information, as well as familial health status were acquired in a questionnaire-based interview. Recruitment was population based; a specific health condition was not required for involvement.

Study groups were selected from all 7936 participants recruited for the LGDB project from 2003 to 2008. We initially excluded patients with cancer, renal diseases, variant angina (ICD-10 I20.1), cardiomyopathy (ICD-10 I42 and I43), and rare diseases (a complete list is available upon request). Participants with missing phenotypic data analyzed in this study were also excluded.

After these exclusions were carried out, patients with CHD manifestations including angina (ICD-10: I20) and MI (ICD-10: I21 and I25.2) were selected for the case group. The final number of patients was 685. To avoid selection bias based on the hospital or a specific disease group, controls were randomly selected only from LGDB participants recruited by general practitioners. Patients with type-2 diabetes (ICD-10:E11) were not excluded from either case or control group. A total of 830 controls without cancer, CHD and its manifestations, renal or hepatic conditions, or thyroid disease were used in the present study.

Information about smoking status, alcohol intake, and physical activity were self-reported. Individuals were designated “smokers” if they have smoked ≥5 cigarettes a day for ≥12 months. Former smokers and current smokers were classified according to these criteria. Physical activity was classified according to self-reporting of previous physical activity. The nonconsumers group comprised individuals who had the following behaviors: not consuming alcohol; consuming alcohol less than once a week; or drinking ≤1.5 units of alcohol per week. The remainder of the group was termed “alcohol consumers.”

The study protocol was approved by Central Medical Ethics Committee of Latvia. Written informed consent was obtained from all participants.

SNP selection and genotyping

The aim of the study was to evaluate the role of nonsynonymous SNPs in *ADORA3* with respect to susceptibility to CHD. Four nonsynonymous SNPs have been detected in *ADORA3* and are listed in the SNP database of the National Center for Biotechnology Information (dbSNP, build 131): rs55653224, rs35986308, rs35511654, and rs2800889. We considered only SNPs that were present in a Caucasian population with a minor allele frequency (MAF) of ≥0.01. Only rs35511654 corresponded to the aforementioned criteria and was therefore selected for analyses.

DNA samples were aliquoted from storage tubes into 96-well polymerase chain reaction (PCR) plates using a Tecan Freedom Evo system (Tecan, Männedorf, Switzerland) with disposable filter tips. DNA concentration was normalized to 7 ng/μL. Genotyping was carried out using an Applied Biosystems TaqMan SNP Genotyping Assay with a modified protocol using 4.75 μL TaqMan Genotyping Mix (Applied Biosystems, Foster City, CA), 0.25 μL SNP genotyping assay ID C_25626674_20 (Applied Biosystems) and 5 μL Millipore H_2O (Millipore, Bedford, MA) on a 7500 Real-Time PCR system (Applied Biosystems). AutoCaller 1.1 (Applied Biosystems) software was used to simultaneously assign genotype calls for all samples. Samples with unsuccessful genotyping were removed. A total of 683 cases and 826 controls were included in the statistical analyses of the study.

Statistical analyses

Statistical analyses were undertaken with the PLINK 1.06 software (http://pngu.mgh.harvard.edu/purcell/plink/) (Purcell *et al.*, 2007). Deviation from the Hardy–Weinberg equilibrium was assessed by the exact test described by Wigginton *et al.* (2005), which is more accurate for rare genotypes. The Cochran–Armitage trend test was used for unadjusted association analysis. Logistic regression assuming an additive, dominant, or recessive mode of inheritance was employed to adjust the analysis for other nongenetic factors. Sex, age, body mass index (BMI), diabetes, smoking, alcohol consumption, and physical activity were included in the logistic regression models as cofactors. The permutation test was carried out using label-swapping between the SNP and trait condition but leaving intact correlation with other covariates; 100,000 permutations were undertaken for each analysis. The chi-square test and Student’s *t*-test were used to compare characteristics between case and control groups. Statistical power was calculated using Quanto software (Natara Software, Naperville, IL) (Gauderman and Morrison, 2006). Our sample size provided 80% power (at $\alpha = 0.05$) to detect an odds ratio (OR) of 1.56 (0.64), assuming an MAF of 0.093 (based on controls).

Results

The general characteristics of the study population are shown in Table 1. Smoking was positively correlated with CHD status ($p < 0.0001$), whereas regular consumption of alcohol showed a negative correlation ($p < 0.0001$). A statistically significant difference in physical activity ($p = 0.12$) between the case and control groups was not observed.

From all exonic SNPs reported from the available databases, rs35511654 was the only nonsynonymous SNP in the A_3R with an MAF >0.01 and was therefore included in the study.

The prevalence of genotyping success was 99.6%. Genotyping was unsuccessful for two CHD patients and four controls. There were no significant deviations from Hardy–Weinberg

TABLE 1. CHARACTERISTICS OF PATIENTS WITH CORONARY HEART DISEASE COMPARED WITH CONTROLS

Characteristic	*Controls* (n = 826)	*Cases* (n = 683)	p
Men, *n* (%)	429 (51.9%)	420 (61.5%)	0.05
Mean age (±SD), years	52.3 ± 13.7	59.3 ± 9.3	< 0.0001
Mean BMI (±SD), kg/m^2	26.83 ± 4.95	28.88 ± 4.97	< 0.0001
Presence of type-2 diabetes, *n* (%)	91 (11.0%)	62 (9.1%)	0.30
Smoking *n* (%)	440 (53.3%)	487 (71.3%)	< 0.0001
Regular alcohol consumption, *n* (%)	190 (23.0%)	63 (9.2%)	< 0.0001
Physically active, *n* (%)	156 (18.9%)	107 (15.7%)	0.12
Presence of angina, *n* (%)	—	601 (88%)	—
Presence of MI, *n* (%)	—	418 (61.2%)	—
Presence of heart failure, *n* (%)	—	443 (64.9%)	—
rs35511654 CC/CA/AA, *n*	5/144/677	3/86/594	—
Minor allele frequency (%)	9.3	6.7	0.009
HWE test *p*-value	0.55	0.53	—

Smoker: current or former smoker who smoked regularly for ≥12 months. Regular consumer of alcohol: individuals consuming alcohol more than once every week and people consuming ≥1.5 units of alcohol every week. Physically active: individuals carrying out a sporting activity >4 times a week; physically demanding daily job; sports professional.

MI, myocardial infarction; CHD, coronary heart disease; BMI, body mass index; SD, standard deviation; HWE, Hardy–Weinberg equilibrium.

equilibrium either in cases or controls or in the subgroups of any condition analyzed (Table 1).

The MAF of rs35511654 observed in our study (0.093) was lower than that reported in the HapMap project for this SNP in a population with Northern and Western European ancestry (0.167).

We observed a significantly decreased frequency of the rs35511654 C allele in the group of CHD patients compared with controls (Table 1). The calculated OR for allele number indicated a protective effect of the C allele against CHD events: OR = 0.70 (95% confidence interval: 0.54–0.91). rs35511654 was significantly associated with the presence of CHD in our study group under the unadjusted Cochran–Armitage test ($p = 0.009$). To remove bias from the confounding factors, we adjusted the association of rs35511654 with CHD by including sex, age, BMI, diabetes, smoking, alcohol consumption, and physical activity in a logistic regression model. A significant difference in genotype distributions between cases and controls was found under the additive and dominant models (Table 2). To minimize the false-positive results, given the relatively small sample size, we repeated the aforementioned analyses carrying out a permutation test with a label swapping method for both analyses using identical parameters. Corrected empirical *p*-values generated by the permutation test remained significant in both models (Table 2).

Discussion

In the present study, we showed the association of a nonsynonymous SNP in *ADORA3* with CHD in a Latvian population. Our results suggested that the minor allele C of rs35511654 is protective for CHD. To increase the power of the study, we included all available participants that matched selected phenotypic criteria, resulting in significant differences between cases and controls with respect to sex and age. We therefore adjusted all statistical analyses using sex and age as covariates in logistic regression models. Interestingly, type-2 diabetes in controls was present at a slightly higher frequency (0.11) compared with cases (0.09). This frequency was not significantly different from the general prevalence in the Latvian population (0.099; unpublished results from the Latvian Society of Endocrinology).

The MAF observed in controls (0.093), when compared with published results, was similar to that of the Finnish population (0.094), but considerably lower than that in the populations of Denmark (0.144) and France (0.158) (Tregouet *et al.*, 2008).

rs35511654 changes isoleucine at position 248 of the receptor to leucine. This amino acid is located in the sixth transmembrane domain of the receptor. It has been shown that nearby amino acids are involved in the recognition of agonists and antagonists (positions 250 N and 247 S, respectively) as well as signal transduction and receptor activation (positions 243 W and 239 E, respectively) (Gao *et al.*, 2002). Changes in amino acids at position 248 may therefore lead to internal structural changes that could affect ligand binding and/or signal transduction properties of the A_3R. Although uncommon, such changes could lead to enhanced ligand-mediated activity or partial constitutive activity of the receptor, thereby enhancing the cardioprotective properties of the A_3R.

The protective properties of minor variants could be due to direct (adenosine A_3R in the heart), indirect (adenosine A_3R in other parts of cardiovascular system or cells of the

TABLE 2. ASSOCIATION OF CORONARY HEART DISEASE WITH RS35511654

	Genetic model	*Number* Cases (n = 683)	Controls (n = 826)	p^a	p^a (100,000 permutations)	*OR (CI 95%)*
CHD versus controls	Additive (CC/CA/AA)	3/86/594	5/144/677	0.025	0.025	0.71 (0.53–0.96)
	Dominant (CC + CA/AA)	89/594	149/677	0.022	0.022	0.70 (0.51–0.95)
	Recessive (CC/AC + AA)	3/680	5/821	0.75	0.77	0.76 (0.15–3.97)

[a]Adjusted for sex, age, BMI, type-2 diabetes, smoking, alcohol consumption, and physical activity.
OR, odds ratio; CI, confidence interval.

immune system), as well as combined action. The relatively high frequency of nonsynonymous SNP may support the protective nature of this polymorphism. CHD manifestations usually occur in later life (usually postreproductive) and positive evolutionary pressure in a population would be rather weak. Studies in murine models revealed that slight overexpression of adenosine A_3R enhances cardioprotection with no adverse effect, but sixfold overexpression in mice resulted in dilated cardiomyopathy (Black *et al.*, 2002). We did not observe a different allele dosage effect in our study group. The presence of a minor allele could also indirectly influence protection of the myocardium via an A_3R-mediated role in the inflammation process. Conflicting information exists regarding the role of the A_3R in the release of inflammatory factors from monocytes (Le Vraux *et al.*, 1993; Zhang *et al.*, 2005). However, it has been shown that adenosine acting through the A_3R inhibits the release of TNF-α from monocytes that propagate formation of plaque lesions in artery walls after differentiating into macrophages. Inhibition of TNF-α release may therefore reduce the rate of growth and the number of plaques. In this way, the variant receptor may contribute to cardioprotection against CHD by reducing the severity of atherosclerosis and slowing its progression.

Our results showed opposite effects compared with those of Tang *et al.* (2007), in which the presence of the C allele was associated with an increased size of infarct. However, there were significant differences with respect to study design and patients groups used between their study and the present study. Our study was population based and represented CHD events without specific selection, whereas they studied a group of patients who had MI only in the left ventricle detected by SPECT. Therefore, the results of these two studies are not directly comparable. One may speculate that the presence of the C allele does not directly contribute to the severity of MI but instead provides better survival in cases of increased infarct size, thus explaining the increased frequency of this allele in patients with more severe forms of CHD. A cohort study with registered fatal events would be needed to test this hypothesis.

In summary, our results showed an association of a nonsynonymous SNP in *ADORA3* that may affect the progression of CHD in a Latvian population.

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Disclosure Statement

No competing financial interests exist.

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3.2 Identification of glyoxalase 1 polymorphisms associated with enzyme activity

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Identification of glyoxalase 1 polymorphisms associated with enzyme activity

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ABSTRACT

The glyoxalase system and its main enzyme, glyoxalase 1 (GLO1), protect cells from advanced glycation end products (AGEs), such as methylglyoxal (MG) and other reactive dicarbonyls, the formation of which is increased in diabetes patients as a result of excessive glycolysis. MG is partly responsible for harmful protein alterations in living cells, notably in neurons, leading to their dysfunction, and recent studies have shown a negative correlation between GLO1 expression and tissue damage. Neuronal dysfunction is a common diabetes complication due to elevated blood sugar levels, leading to high levels of AGEs. The aim of our study was to determine whether single nucleotide polymorphisms (SNPs) in the *GLO1* gene influence activity of the enzyme. In total, 125 healthy controls, 101 type 1 diabetes, and 100 type 2 diabetes patients were genotyped for three common SNPs, rs2736654 (A111E), rs1130534 (G124G), and rs1049346 (5′-UTR), in *GLO1*. GLO1 activity was determined in whole blood lysates for all participants of the study.

Our results showed a significant association between the minor alleles rs1130534 and rs1049346 and decreased enzyme activity ($P=0.001$ and $P=2.61\times10^{-5}$, respectively). Increased allelic counts of the risk alleles were strongly associated with decreased GLO1 activity (standardised $\beta=-0.24$, $P=2.15\times10^{-5}$), indicating independent actions of these variants on GLO1 activity, as supported by the haplotype analysis. We showed for the first time an association between genetic variants with GLO1 enzyme activity in humans. SNPs in *GLO1* can be used to predict enzyme activity and detoxifying capabilities, but further studies are needed to link these SNPs with common complications in diabetes.

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1. Introduction

The glyoxalase system, including the enzymes glyoxalase 1 (EC 4.4.1.5, GLO1) and glyoxalase 2 (EC 3.1.2.6, GLO2), protects living cells from producing and accumulating advanced glycation end products (AGEs). GLO1 is the main enzyme in this system, being responsible for converting reactive dicarbonyls into non-toxic intermediates (Thornalley, 1990). The importance of GLO1 is supported by phylogenetic conservation in various taxa, such as protozoa, fungi, bacteria, plants, and mammals, including humans (Thornalley, 2003). The glyoxalase system classically has been studied in context of diabetes, characterised by elevated glucose levels. Today, there is overwhelming evidence for an association between AGEs and highly reactive dicarbonyl metabolite methylglyoxal (MG) and increased diabetes complications in humans and diabetic mice/rat models, including neuropathy, endothelial damage of blood vessels, retinopathy, nephropathy, and premature ageing (Berner et al., 2012; Jack et al., 2012; Morcos et al., 2008; Wautier and Schmidt, 2004). It has recently been shown that MG induces primary hyperalgesia in animals and could be a cause of pain in patients with diabetes, by post-translationally modifying the nociceptor-specific sodium channel $Na_v1.8$ (Bierhaus et al., 2012). GLO1 has also been investigated in anxiety, including a murine model showing that gene overexpression increases anxiety-related behaviour Hovatta et al.

Abbreviations: 5′-UTR, 5 prime untranslated region; ACE inhibitor/ARB, angiotensin converting enzyme inhibitors and angiotensin receptor blocker; AGE, advanced glycation end product; BMI, body mass index; EMP2, corrected empirical p-value; GABA, gamma-aminobutyric acid; GLO1, glyoxalase 1; GLO2, glyoxalase 2; Hap, haplotype; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; LD, linkage disequilibrium; LDL, low-density lipoprotein; MAF, minor allele frequencies; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometer; MG, methylglyoxal; SD, standard deviation; SNP, single nucleotide polymorphism; T1D, type 1 diabetes; T2D, type 2 diabetes; U/g Hb, units per gram hemoglobin.

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(2005) by reducing the $GABA_A$ receptor agonist MG (Distler et al., 2012). An association analysis in a human population with anxiety disorder subtypes suggested a role ($0.01<P<0.05$) for GLO1 involvement in this phenotype (Donner et al., 2008). GLO1 appears to have an important role in tumour growth and partly in chemotherapy survival. A number of studies suggests that some tumours rely on GLO1 to increase their viability and resistance to multiple drugs (Thornalley and Rabbani, 2011).

Several publications have studied the association of single nucleotide polymorphism (SNP) rs2736654 (also known as rs4746) (A111E), in *GLO1* and autism with inconclusive results in different populations (Junaid et al., 2004; Rehnstrom et al., 2008). Glutamic acid at the 111 position of GLO1 reduces the activity of the enzyme in immortalized lymphoblastoid cells (Barua et al., 2011). However, the A allele of rs2736654 was not associated with nephropathy, retinopathy, or an altered albumin/creatinine ratio in a Chinese population (Wu et al., 2011). rs1049346, located in the 5′-UTR of *GLO1*, was associated with type 2 diabetes complications: namely, retinopathy and nephropathy (Wu et al., 2011). Although AGEs are known to damage blood vessels, no link between polymorphisms in *GLO1* and vascular complications was found in a cohort of European patients (Engelen et al., 2009). In this study, we estimated the influence of three *GLO1* polymorphisms on enzyme activity.

2. Methods

In total, 326 participants were recruited by the Latvian Genome Database (LGDB), a government funded biobank (shortly described in Ignatovica et al. (2012)) from a recent study of GLO1's association with painful neuropathy (Skapare et al., in press). This case-control study included 101 patients with type 1 diabetes (T1D), 100 patients with type 2 diabetes (T2D), and 125 non-diabetic individuals. All control participants and patients were of European descent.

Exclusion criteria were chronic renal failure (glomerular filtration rate<60 ml/min), chronic liver disease, an active inflammatory disorder (white blood cell count$>8.5\times10^3/mm^3$), an erythrocyte sedimentation rate>20 mm/h, or high-sensitivity C-reactive protein (hs-CRP)>15 mg/l (because high levels may indicate a latent chronic inflammatory condition), anaemia, current evidence or a history of malignancy in the last 5 years, a history of drug or alcohol abuse, and causes of neuropathy other than diabetes. Data collected at study entry included age, medical history, age at diagnosis, medication, smoking history, and alcohol intake.

The study was carried out in accordance with the Declaration of Helsinki and was approved by the Central Medical Ethics Committee of Latvia (Resolution A-9, May 29, 2006). Written informed consent was obtained from all participants.

We selected three SNPs (rs2736654, rs1130534, rs1049346) in the *GLO1* gene using the following selection criteria: SNPs located inside the coding region or in close proximity (within 0.1 kb) of *GLO1*, and SNPs with minor allele frequencies (MAF) above 0.1 in the Caucasian population, based on information in the NCBI SNP database.

Genotyping was performed by minisequencing and subsequent MALDI-TOF mass spectrometry, as described previously (Ignatovica et al., 2012). Primer sequences are available on request.

Analysis of results was performed using the Genotools SNP Manager Software (Bruker Daltonics, Germany) and verified manually afterwards. GLO1 activity was determined in whole blood samples on the day of the blood collection, according to a method described previously (Skapare et al., 2011). Statistical analyses were performed using the PLINK 1.07 software (http://pngu.mgh.harvard.edu/purcell/plink/) (Purcell et al., 2007). Deviation from Hardy-Weinberg equilibrium was assessed using the exact test. Blood GLO1 activity values were distributed normally, as determined using the Kolmogorov–Smirnov test. Linear regression was used to determine relationships between genotypes and GLO1 enzyme activity with and without covariates (gender, age, BMI, smoking, alcohol consumption, history of statin and ACE inhibitor/ARB therapies). To minimize the false-positive results, given the relatively small sample size, we carried out a permutation test with a 'label swapping' method using identical parameters; 100,000 permutations were used including the correction for multiple testing (EMP2). The *t*-test was used to compare means between genotypes and weighted averages are reported. Haplotype association was performed as implemented in PLINK. *P* values<0.05 after adjustments were considered to indicate statistical significance.

3. Results

General characteristics of the study population are shown in Table 1. The mean age of the study group was 46.3 ± 5.3 years. Total genotyping success was 99.7%. rs1130534 and rs2736654 were successfully genotyped in all study subjects, while three genotypes for rs1049346 were not acquired. No deviation from Hardy-Weinberg equilibrium was observed for any of the SNPs ($P>0.05$). We observed significantly decreased blood GLO1 activity associated with the presence of rs1130534 AT and TT and rs1049346 TT and CT genotypes (Table 2). For each T allele of rs1130534 there was a decrease of blood GLO1 activity of an average of 3.1 U/g Hb, while a decrease in activity of an average of 2.8 U/g Hb was observed for each T allele of rs1049346. The results remained significant after a permutation test and correction for multiple testing. Linkage disequilibrium (LD) calculations revealed strong linkage disequilibrium between the two SNP pairs (Fig. 1). Haplotype reconstruction analysis identified five common haplotypes with minor haplotype frequencies>0.01 when all three SNPs were included. A summary of haplotype-based association results with regard to GLO1 activity is presented in Fig. 1. Haplotype analysis results showed the significant impact of two SNPs, rs1130534 and rs1049346, on GLO1 activity with probable action of rs2736654 as a modulator. Individuals carrying minor alleles in both rs1130534 and rs1049346 loci (Hap2) had significantly lower GLO1 activity (21.8 ± 4.3 U/g Hb) than individuals with both major alleles in the same positions (33.0 ± 7.0 U/g; Hap4). At the same time, the presence of the rs2736654 C allele lowered the GLO1 activity by 0.8 U/g Hb (Hap1, Hap4), compared with the respective haplotypes containing the A allele (Hap3 and Hap5, respectively). When analysed independently, an increased number of risk alleles was associated significantly (standardized $\beta=-0.24$; $P=2.15\times10^{-5}$) with decreased GLO1 activity (Fig. 2). No association was observed for any of these

Table 1
Description of 326 study participants.

Characteristic, unit	Value
Age, years ± SD	46.3 ± 15.3
Male sex, *n* (%)	139 (43%)
Type 1 diabetes, *n* (%)	100 (31%)
Type 2 diabetes, *n* (%)	101 (31%)
Fasting plasma glucose, mmol/l	7.41 ± 3.10
HbA_{1C}, % ($N=230$)	8.23 ± 2.27
BMI, kg/m^2	27.6 ± 5.9
hs-CRP, mg/l ($N=257$)	2.22 ± 2.16
Total cholesterol, mmol/l	5.58 ± 1.16
Triacylglycerol, mmol/l	1.87 ± 1.65
HDL-cholesterol, mmol/l ($N=165$)	1.45 ± 0.45
LDL-cholesterol, mmol/l ($N=172$)	3.51 ± 1.02
C-peptide, ng/ml ($N=260$)	1.90 ± 1.72
Blood GLO-1 activity, U/g Hb	31.39 ± 8.00
Current smoker, *n* (%)	95 (29%)
Alcohol intake (≥1 consumptions/week) (%)	33 (10%)
Statin therapy, *n* (%)	94 (29%)
ACE-inhibitor/ARB therapy, *n* (%)	144 (44%)
rs1130534 MAF (T)	0.15
rs2736654 (rs4746) MAF (C)	0.42
rs1049346 MAF (T)	0.49

Table 2
GLO1 polymorphisms associated with GLO1 activity.

	Genotype	*n* (%)	Blood GLO1 activity, U/g Hb	*P*	*P*[a]	*P* 100,000 permutations[a]
rs1130534	AA	234 (72)	31.6 ± 8.5			
	AT	86 (29)	28.6 ± 8.3	**0.001**	**0.004**	**0.01**
	TT	6 (2)	25.2 ± 10.0			
rs2736654 (rs4746)	AA	105 (32)	31.1 ± 8.8			
	AC	168 (52)	30.9 ± 9.2	0.30	0.47	0.83
	CC	53 (16)	29.4 ± 5.4			
rs1049346	TT	80 (25)	27.2 ± 8.0			
	CT	158 (49)	31.4 ± 9.1	$\mathbf{2.61 \times 10^{-5}}$	**0.0001**	**0.0003**
	CC	85 (26)	32.8 ± 7.1			

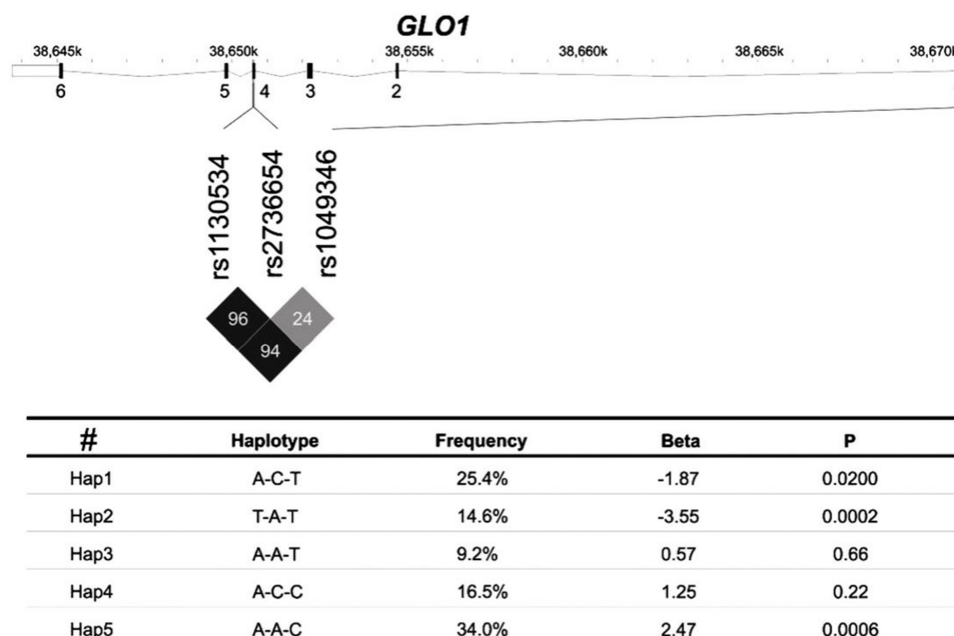
[a] *P* value adjusted using covariates sex, age, BMI, smoking, alcohol consumption, statin and ACE inhibitor/ARB therapies. *P* values <0.05 after 100,000 permutations were considered to indicate statistical significance.

SNPs with presence of diabetes (T1D or T2D, or both together) in our sample group (data not shown).

4. Discussion

The connection between reactive dicarbonyls, such as MG, AGEs, the glyoxalase system, and complications of diabetes has been the subject of intensive research (Thornalley, 1990, 2003; Wautier and Schmidt, 2004; Wu et al., 2011). Our results suggest for the first time an association between the T allele of rs1049346, located in the 5′-UTR, and the T allele of synonymous SNP rs1130534 with decreased activity of GLO1 measured in whole blood samples. The association between genetic variations in *GLO1* with altered enzyme activity was more pronounced with haplotypes consisting of minor alleles of two SNPs, rs1130534 and rs1049346.

The main strengths of our study are the well-characterized study group, established GLO1 enzyme activity assay, and recognized genotyping method. Due to the small sample size, which is the main limitation of the study, we focused our research on detecting the influence of a few frequent SNPs directly on enzyme activity rather than trying to link SNPs and/or haplotypes with phenotypes that could arise due to altered GLO1 activity. Historically, one of the first SNPs to be described in *GLO1* was rs4746 (re-named as rs2736654) that changes alanine to glutamic acid at amino acid position 111 (Kompf et al., 1975). It has been shown that glutamic acid in 111 position of *GLO1* reduces the activity of enzyme in immortalized lymphoblastoid cell lines Barua et al. (2011), while our study results revealed that it had the opposite – although not statistically significant – effect on GLO1 activity estimated from whole blood lysate. This difference may be explained by the differing experimental designs. One may expect a more pronounced effect of a non-synonymous change when cultured lymphoblastoid cells are used instead of whole blood lysate. On the other hand, two other SNPs (rs1130534 and rs1049346) were not analysed in the study of Barua et al. (2011) and it would be interesting to observe the effect of these SNPs on GLO1 activity. Another difference is that our study was performed using a relatively large group of unrelated individuals, compared with five cell lines per genotype originating from an autism study including some families. It may be that the observed effect could be explained by the presence of two other common SNPs (rs1130534 and rs1049346) that are in linkage disequilibrium with rs2736654 and



#	Haplotype	Frequency	Beta	P
Hap1	A-C-T	25.4%	-1.87	0.0200
Hap2	T-A-T	14.6%	-3.55	0.0002
Hap3	A-A-T	9.2%	0.57	0.66
Hap4	A-C-C	16.5%	1.25	0.22
Hap5	A-A-C	34.0%	2.47	0.0006

Fig. 1. Haplotype association results. A schematic representation of a GLO1 gene and a pairwise LD plot with D' values. Table lists results of a haplotype association with GLO1 activity. Haplotypes exceeding 1% frequency are shown.

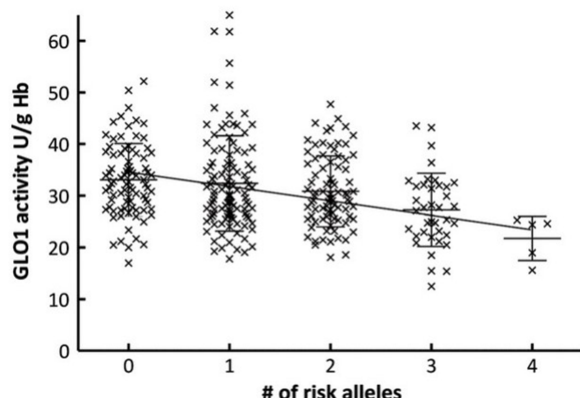


Fig. 2. Risk allele count effect on GLO1 activity showing an association between increased number of risk alleles per individual and decreased GLO1 activity. Two statistically significantly associated SNPs (rs1130534 and rs1049346) presented.

are, in fact, associated with GLO1 activity. An independent effect of rs2736654 also cannot be ruled out, because our haplotype analysis showed that the presence of a C allele decreased the mean GLO1 activity of these haplotypes (Fig. 1). An independent effect of each of the variants studied is further supported by linear regression analysis showing a remarkable decrease in GLO1 activity with increase in number of risk alleles per individual (Figs. 1, 2). Similarly, epistasis may possibly occur, where the effect of enzyme isoforms may be enhanced or silenced by the presence of variation that affects the expression of the protein. Regarding the functionality of variants analysed, it should be noted that two of the SNPs tested do not change the amino acid sequence of GLO1. rs1049346 is located in the 5 -UTR and may be involved in disrupting the regulation of expression, resulting in a decrease in enzyme activity. The possible functional involvement of rs1130534 in relation to decreased GLO1 activity is unclear; however, there are convincing results on the role of other synonymous SNPs in altered phenotypes due to disrupted gene regulation (Brest et al., 2011). Further functional studies including estimation of mRNA and/or protein levels are needed to confirm the functionality of the polymorphisms studied. It should be also noted that effects of GLO1 activity changes observed in blood cannot be directly extrapolated to other organs where specific factors may modulate the enzymatic activity versus blood cells.

We did not observe an association between the SNPs and the presence of diabetes corresponding to data from various genome-wide association studies of diabetes (Saxena et al., 2007). In a mouse model, overexpression of GLO1 may protect from peripheral sensory neuron damage Jack et al. (2012), but whether the same is true in humans and whether polymorphisms in *GLO1* pose a risk to peripheral neurons remain unclear. Thus, no association between *GLO1* non-synonymous polymorphism rs2736654 and diabetic complications was observed by Wu and colleagues while a positive association was found with rs1049346 and retinopathy and nephropathy. Further studies with detailed information on the presence of diabetic complications and increased numbers of subjects are necessary to investigate the role of these variations in increasing vascular complications in diabetic patients. Our study provides key insights into common genetic factors that influence GLO1 activity, but not the presence of diabetes in our study group.

Acknowledgments

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3.3 Polymorphisms in MEN1 and DRD2 genes are associated with the occurrence and characteristics of pituitary adenomas

Polymorphisms in *MEN1* and *DRD2* genes are associated with the occurrence and characteristics of pituitary adenomas

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Abstract

Objective: Although pituitary adenomas (PAs) affect a significant proportion of the population, only a fraction have the potential to become clinically relevant during an individual's lifetime, causing hormonal imbalance or complications due to mass effect. The overwhelming majority of cases are sporadic and without a clear familial history, and the genotype–phenotype correlation in PA patients is poorly understood. Our aim was to investigate the involvement of genes known for their role in familial cases on drug response and tumor suppression in the development and pathology of PAs in a patient group from Latvia.
Design: The study included 143 cases and 354 controls, we investigated the role of single-nucleotide polymorphisms (SNPs) in seven genes (*SSTR2*, *SSTR5*, *DRD2*, *MEN1*, *AIP*, *GNAS*, and *PRKAR1A*) associated with pituitary tumor occurrence, phenotype, and clinical symptoms.
Methods: Genotyping of 96 tag and nonsynonymous SNPs was performed in the genomic regions of interest.
Results: We discovered a significant association (OR = 17.8, CI 0.95 = 2.18–145.5, $P = 0.0002$) between a rare *MEN1* mutation (rs2959656) and clinically active adenoma in our patients. Additionally, rs7131056 at *DRD2* was associated with a higher occurrence of extrasellar growth in patients with prolactinoma and somatotropinoma (OR = 2.79, CI 0.95 = 1.58–4.95, $P = 0.0004$).
Conclusions: rs2959656, a nonsynonymous variant in *MEN1*, is associated with the development of clinically active PA. Furthermore, rs7131056 in *DRD2* contributes to either faster growth of the adenoma or reduced symptomatic presentation, allowing PAs to become larger before detection.

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Introduction

Up to 15% of clinically active primary intracranial neoplasms are pituitary adenomas (PAs) (1). Clinically significant PAs affect one individual out of approximately 1000–1300 people in the general population (2, 3). Silent adenomas are more common and are found in 1.5–27.0% (mean 10.7%) of people examined (4). Meta-analysis estimation shows a prevalence of 16.7% in the general population (5). PAs are categorized by their size and type of hormone secreted. The most common type of clinically significant PAs is prolactin-secreting (40% of all PAs), followed by nonfunctioning PAs (NFPAs) (37%) and growth hormone (GH)-secreting (somatotroph) adenomas (13%) (6). It was formerly thought that PA-caused acromegaly had a prevalence of 40–60 (7, 8) patients per million people, but more recent findings show that clinically relevant somatotroph adenomas present a rate of 124/1 000 000 (9) people in Europe and 480/1 000 000 (10) in Brazil. More than 70% of GH-secreting adenomas are larger than 10 mm in diameter (macroadenomas) at the time of diagnosis (11), probably because tumor formation can start as many as 10 years before diagnosis (7).

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There is evidence that both genetic and epigenetic factors are important for the development of human neoplasms (12). It has been shown that PAs are tumors of monoclonal origin (13). At least three separate clinical syndromes have been associated with hereditary PAs, including multiple endocrine neoplasia type 1 (MEN1), Carney complex, and familial isolated PA, caused by mutations in *MEN1*, *PRKAR1A*, and *AIP*, respectively (14, 15, 16). About 5% of PAs can be attributed to these familial syndromes, while others are considered as sporadic cases thought to be caused by mutations in various genes including tumor suppressors in the retinoblastoma tumor suppressor and p53 pathways (17). Research shows that a wider range of clinically significant adenomas tend to aggregate in families (18). The genetic mechanisms underlying common forms of adenoma are less clear, with multiple genes potentially involved in the pathogenicity of PA. A recent genome-wide association study identified two markers on chromosome 10 and one on chromosome 13 to the presence of sporadic mutations in the Han Chinese population (19). A novel finding suggests Xq26.3 microduplication responsible for sizeable proportion of the infantile pituitary gigantism cases, and further examination of the genes in this region in sporadic acromegaly cases suggests *GPR101* as a cause (20). A MEN1-like syndrome (MEN4) has been described more recently in rats and humans and relates to mutations in the *CDKN1B* gene, which may be considered as a rare cause of pituitary adenomas (21). *AIP* mutations, normally associated with familial isolated PA, have also been shown to be rarely involved in the occurrence of sporadic cases (22, 23) and lack of response to somatostatin analogs (SA) in acromegaly (18, 23, 24). Somatostatin acting through somatostatin receptors (SSTRs) is an important mediator of GH secretion and regulation of tumor proliferation; therefore, this system has been extensively studied in acromegaly (25, 26, 27), and our previous study identified an association between polymorphisms in *SSTR5* with acromegaly and number of disease parameters (28). Similarly, the involvement of the dopamine system in clinically significant PA has been suggested, since dopamine agonists inhibit production of prolactin from the pituitary gland (29). The targeting of *DRD2* with dopamine agonists has been used successfully in patients with elevated hormone levels in prolactin-secreting and/or mixed GH/prolactin adenomas (30). Somatic mutations in the Gsα gene (*GNAS*) are found in 40% of GH-secreting PAs, resulting in increased sensitivity to the inhibitory action of somatostatin (31).

In this research article, we present results from genotyping of tag single-nucleotide polymorphisms (SNPs) for seven candidate genes (*AIP*, *SSTR2*, *SSTR5*, *DRD2*, *GNAS*, *MEN1*, and *PRKAR1A*) and their neighboring regions (in strong linkage disequilibrium (LD) with markers within the gene) in samples from 143 sporadic PA patients and 354 age- and sex-matched controls (at a 1:2.5 ratio of cases to controls) in order to investigate the potential involvement of these genes in nonfamilial PA cases.

Methods

Study population and diagnosis

This study was carried out using DNA samples from the Latvian Genome Database (LGDB) (briefly described previously) (32). DNA was extracted from the whole-blood leukocytes by phenol–chloroform method. Written informed consent was acquired from all LGDB participants. The study protocol was approved by the Central Medical Ethics Committee of Latvia (protocol no. 01.29.1/28, 14 December 2011). The case group (165 participants) was enrolled for this study from the LGDB between 2004 and November 2011. Two university hospitals involved in the treatment of PA in Latvia participated in the enrollment of study cases. One hundred and fifty-eight patients were recruited from Pauls Stradiņš Clinical University Hospital and seven patients were from Riga Eastern Clinical University Hospital. Hormonal profiles (typically levels of GH, prolactin, insulin-like growth factor 1 (IGF1), adrenocorticotropic hormone (ACTH), and others, depending on the presence of clinical indications) from the blood were determined at an independent commercial laboratory (E. Gulbis Laboratory, Riga, Latvia). Prolactin was detected by electrochemical luminescence (Cobas 6000, Roche), while GH, IGF1, and ACTH levels were measured with Imulite 2000 (Siemens AG, Berlin, Germany). In cases with elevated levels of these hormones, magnetic resonance imaging (MRI) was performed. The presence of a pituitary tumor was determined using high-definition MRI data from a 1 mm slice series of the pituitary gland without contrasting agent with a Siemens Magneton 1.5T (Siemens AG, Berlin, Germany). During the first visit to the endocrinologist, phenotypic features (primary diabetes, primary hypertension, heart dysfunction, carpal bone syndrome, sleep apnea, macrognathia, and morphology of the nose and frontal bone) were also considered in the diagnosis, and further blood serum hormone testing was performed. Anthropometric and social data,

health records, and family histories were obtained when including the patient in the study. Data used in this study include sex, age, age at tumor diagnosis, PA size, extrasellar growth, and hormone-secreting type, treatment type, drug prescription information, and the occurrence of other tumors. PA was designated as a "macroadenoma" if its diameter according to the MRI data was larger than 10 mm. It must be noted that age at diagnosis does not correspond to the age of tumor incidence or age when clinical symptoms attributed to PA occurred. Phenotypic data were collected based on hospital records and interviews for all the patients selected for the study. Samples were excluded from the study due to missing phenotype data (age or sex; $N=9$), misdiagnosed as having PA in the primary examination ($N=5$), inadequate DNA sample quality ($N=2$), or rediagnosis as a pituitary carcinoma ($N=1$). Due to the low number of cases, we also excluded patients with Cushing's disease ($N=4$), leaving 144 cases eligible for genotyping.

Age- and sex-matched controls were selected from LGDB participants without endocrine or metabolic diseases or other chronic disease. Matching of samples was performed by dividing the case sample into groups based on sex, then further dividing subjects into age bins by decade. The sample of suitable controls ($N=738$) was divided in a similar manner. Controls were picked randomly from each subgroup according to a number based on each respective case group. Samples with phenotypic information missing (e.g., sex, age, or BMI) were excluded from random matching. It should be noted that sex and age matching was performed before genotyping, and thus, it was influenced by exclusion of some samples as a result of genotyping quality check. However, the medians of age and interquartile range remained similar for all respective case–control groups, and in case of linear and logistic regression, sex was used as a covariate controlling for this issue. A total of 365 controls and one interplate positive control were used in this study. The case-to-control ratio was 1:2.5.

Candidate gene and SNP selection

Seven candidate genes were selected based on an extensive literature search about known and probable PA genetics in February 2011. The genes chosen were *AIP, MEN1, GNAS, SSTR2, SSTR5, PRKAR1A*, and *DRD2*. Tag SNPs were selected within these genes and in the upstream and downstream regions in strong linkage disequilibrium (LD) ($r^2>0.8$) with markers within each gene using Haploview v4.2 software (33) and HapMap release #28 (NCBI build 36, dbSNP b126, CEU analysis panel) (34) project information available in February 2011. Nonsynonymous SNPs in these genes were also included, and the remaining two slots in the 96-SNP assay were filled with the most informative tag SNPs from *PRKAR1A*. Detailed information about the regions chosen and LD plots are available upon request. The SNP list generated by Haploview was analyzed by the Illumina Assay Design Tool (Illumina, San Diego, CA, USA). SNPs with low predicted genotyping success rates were removed and each gene was reanalyzed in Haploview using the "force exclude" option for the marked polymorphisms. The designated genotyping success rate of 0.5 was deemed acceptable in cases where no other SNP was present to capture the marker or important nonsynonymous variant selected. Otherwise, a "Designability Rank = 1" was required. The final number of SNPs corresponded to the requirements of the Custom VeraCode GoldenGate Genotyping platform, offering discrimination of 96 SNPs in a single well of a 96-well microplate as one of the standard options. A full list of all SNPs and respective information about each is available upon request.

Genotyping and quality control

All 96 SNPs were genotyped using the Illumina BeadXpress system (Illumina GoldenGate genotyping assay) (35). Genotyping was carried out according to the manufacturer's instructions. In order to ensure quality control (QC) and a high intrasubject concordance rate, 9.3% or 46 DNA samples were randomly chosen and distributed to other plates for repeated genotyping. All QC steps in the manufacturer's instructions were meticulously implemented. One positive sample was used in all plates. There was >99.98% observed concordance; there was one mismatched allele call in 46 repeat pairs (one out of 5796), excluding uncalled SNPs and alleles. Additionally, only one mismatched allele call was observed among seven positive controls; therefore, no assay plates were removed from the study. Primary genotyping data analysis was performed by Illumina GenomeStudio V2010.3 Genotyping module V1.8.4. software. The Gene Call threshold was set to 0.25. The cluster images of the signal intensity were manually reviewed.

Sanger sequencing was performed to confirm rs2959656 heterozygous state in seven carriers. Polymerase chain reaction primers were 5′-AGCCAGCACTGGACAAGG-3′ and 5′-CCTTCATGCCCTTCATCTTC-3′. Sequencing

primers were 5′-GGAAGCCTCCTGGGACTGT-3′ and 5′-TCTGGAAAGTGAGCACTGGA-3′.

Statistical analysis

PLINK v1.07 (36) was used for statistical analyses. A basic association test was used to test the difference between cases and controls. Logistic regression was used to test for a difference between cases and controls, adjusting the results for smoking status and sex. Linear regression was used to test the association with quantitative variables. *P*-values lower than 0.00081 to account for Bonferroni multiple testing correction for 62 SNPs tested were considered significant. Initial *P*-values lower than 0.05 were deemed noteworthy and such results were examined closer.

Statistical power was calculated using Quanto v1.2.4 for SNP with frequencies of 1, 5, 10, and 50% using the assumptions of gene-only hypothesis, log-additive inheritance, and a population risk of 0.001 in a two-tailed test using our sample (Fig. 1).

Age was not normally distributed in the whole case or control groups due to the fact that PAs are age-related and controls were age-matched to cases. However, the quantitative variable 'age at diagnosis' was normally distributed in PA patients and subgroups of different adenoma types according to a chi-square goodness-of-fit test ($P>0.05$). Normally distributed quantitative variables are presented as mean ± s.d. Quantitative variables that do not follow normal distribution are presented as medians and interquartile range (Table 1).

Mann–Whitney rank sum test was used to compare quantitative variables that are not normally distributed.

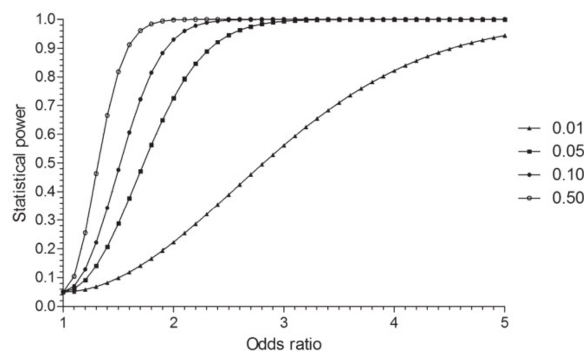

Figure 1
Statistical power calculated for samples in this study (143 cases and 355 controls) at single-nucleotide polymorphism frequencies of 0.01, 0.05, 0.1, and 0.5.

Results

The aim of this study was to genotype a set of 96 SNPs from seven candidate genes, which according to available literature data could be potentially involved in PA in 144 cases and 366 controls. Thirty-three SNPs were excluded from further analysis, with 11 SNPs excluded due to undistinguishable clusters and 22 excluded for being monomorphic (having a single cluster). Twelve samples (11 controls and one case) were excluded due to call rates of less than 90%. One SNP (rs4938025) did not pass the Hardy–Weinberg equilibrium test ($P<0.01$) according to PLINK and was excluded. After QC, 497 individuals genotyped for 62 SNPs remained eligible for further statistical analysis. Detailed characteristics of the study subjects are given in Table 1.

The strongest association signal was observed in *MEN1*. Carriers of the rare nonsynonymous SNP rs2959656 had a significantly increased risk for developing a pituitary tumor (OR = 17.8, $P=0.0002$) (Table 2). Seven heterozygous carriers of this SNP were observed in the case group, while only one 26-year-old individual from the control group had the same variant. The presence of this SNP was not associated with the specific hormonal profile of adenoma. Five of the seven

Table 1 Characteristics of study population.

Variable	Cases	Controls
n	143	354
SNPs analyzed	62	62
PA (overall) male-to-female ratio	1:1.86	–
PA (overall) mean age at diagnosis ± s.d.	44.3 ± 15.9	–
GH-secreting adenomas (*n*)	66	–
GH-secreting adenomas male-to-female ratio	1:2.14	–
GH-secreting adenomas mean age at diagnosis ± s.d.	44.7 ± 14.0	–
Prolactinomas (*n*)	46	–
Prolacinomas male-to-female ratio	1:1.56	–
Prolacinomas mean age at diagnosis ± s.d.	38.9 ± 15.6	–
NFPA (*n*)	31	–
NFPA male-to-female ratio	1:1.82	–
NFPA mean age at diagnosis ± s.d.	51.0 ± 17.6	–
PAs with extrasellar extension (*n*, %)	77 (53.8%)	–
Median age, years (Q1–Q3)	50 (35.5–60.5)	51 (39.75–62.0)
Male sex (*n*, %)	50 (35.0%)	96 (27.1%)
Smoking history (*n*, %)	87 (60.8%)	227 (64.1%)

GH, growth hormone; NFPA, nonfunctioning pituitary adenoma; PA, pituitary adenoma; Q1–Q3, interquartile range; s.d., standard deviation.

Table 2 Association results of SNPs with categorical variables (condition vs control group) (not corrected for multiple testing).

Condition	Gene	SNP	F_PA	F_C	OR (CI 95%)	*P*	*P**
PA	*MEN1*	rs2959656	0.024	0.001	17.8 (2.18–145.5)	0.0002	0.005
GH-secreting PA	*SSTR5*	rs34037914	0.113	0.046	2.63 (1.36–5.08)	0.003	0.006
	SSTR5	rs2076421	0.262	0.363	0.62 (0.41–0.95)	0.03	0.04
	SSTR5	rs169068	0.554	0.456	1.48 (1.01–2.16)	0.04	0.04
Prolactinoma	*MEN1*	rs624975	0.228	0.141	1.80 (1.06–3.06)	0.03	0.05
NFPA	*DRD2*	rs7125415	0.194	0.105	2.06 (1.05–4.04)	0.03	0.05
GH- or prolactin-secreting PA with extrasellar growth	*DRD2*	rs7131056	0.587	0.337	2.79 (1.58–4.95)	0.0004	0.0002
	DRD2	rs4938025	0.585	0.326	2.92 (1.60–5.30)	0.0004	0.002

CI, confidence interval; F_C, SNP frequency in controls; F_PA, SNP frequency in cases; OR, odds ratio; PA, pituitary adenoma; *, adjusted for covariates: sex, BMI, and smoking history.

carriers had clinically significant microadenoma and two others had macroadenomas.

However, several other SNP–phenotype associations with certain types of adenoma or specific disease phenotypes were noted and examined closer. None of these, however, remained statistically significant after Bonferroni correction for multiple testing (correcting for 62 SNPs genotyped). The strongest association trend with acromegaly was rs34037914 at *SSTR5*. A number of disease phenotypes in our study were adenoma-specific or were not equally distributed among different types of tumors. Thus, extrasellar growth of adenomas was more frequently observed in NFPA (77%) compared with both hormone-secreting adenoma groups (46%) ($P=0.004$). Therefore, we assessed the association with this phenotype in these subgroups. While none of the SNPs were associated with extrasellar growth in the NFPA group, two *DRD2* SNPs (rs7131056 and rs4938025) were associated with extrasellar growth in the joint analysis of GH- and prolactin-secreting adenomas. Association results of categorical parameters are given in Table 2.

More closely investigated association results with age at diagnosis are given in Table 3. Four SNPs show association trend with a difference in the age at diagnosis of PA. The minor alleles of three SNPs decreased the age at PA diagnosis by 5–17 years, while the minor allele of *DRD2* variant rs2734849 increased the age at diagnosis by almost 6 years. In the subgroup of cases with acromegaly, two other SNPs show trend of association with decreased age at diagnosis (rs624975 in *MEN1* and rs1800497 in *DRD2*) (Table 3).

Haplotype reconstruction using our genotyping data did not reveal strong LD between the chosen tag SNPs in the Latvian population. Differences in the allele frequencies of SNPs between the Latvian and CEU populations are available upon request.

Discussion

In this study, multiple tag SNPs were genotyped in seven candidate genes in order to investigate their influence on the development of PAs and specific disease phenotypes. Due to the limited sample size, our primary aim was to detect clinically relevant genetic changes with large effects in relation to observed phenotypes (OR>3; $-5>\beta>+5$). Only recently, the first genome-wide association study on PA has been published. In that study, two markers on chromosome 10 and one on chromosome 13 were associated with elevated PA risk in the Han Chinese population. With ORs ranging from 1.28 to 1.44, these SNPs have modest influence on increased risk for PA development (19). Even

Table 3 Top association results of SNPs with quantitative variables (not corrected for multiple testing).

Condition	Gene	SNP	Nc	Nwt	Beta	*P*	*P**
Age at PA diagnosis	*DRD2*	rs2734849	103	40	5.84	0.002	0.001
	DRD2	rs2002453	69	72	−5.06	0.02	0.01
	MEN1	rs607969	4	137	−17.6	0.03	0.03
	SSTR5	rs34037914	18	113	−7.07	0.04	0.04
Age at GH-secreting PA diagnosis	*MEN1*	rs607969	4	62	−19.07	0.007	0.006
	SSTR5	rs34037914	12	50	−9.21	0.009	0.02
	MEN1	rs624975	18	48	−9.12	0.009	0.01
	DRD2	rs1800497	19	27	−8.62	0.013	0.007

Beta, regression coefficient; GH, growth hormone; Nc, number of minor allele carriers; Nwt, number of wild-type carriers; PA, pituitary adenoma; *, adjusted for covariates: sex, smoking history.

if this data were available at the design stage of our study, the chance of detecting effects at these ORs in our limited population size would be low (Fig. 1).

Few candidate gene studies have suggested potential contributors to PA development. For example, *LRRC4* has been shown to have a protective effect for the T allele at rs6944446 (OR=0.44, P=0.036) (37). Another study found that two *CYP2D6* alleles increased susceptibility to pituitary tumors (38).

Recently, there is more attention on research of genetic changes in tumor samples that give insights into mutation rate and mechanisms in formation of PA and their clinical phenotypes (20, 39, 40). Main conclusion from these studies is that mutations detectable with current sequencing technologies in the exome of the somatic cells are zero (40) to seven (41) per tumor and that corresponds to generally slow proliferation of benign pituitary adenomas. However, no recurrent somatic mutations were detected in the exomes of seven NFPAs and 24 controls (41) or 12 genomes of prolactin and GH-secreting Pas (40). The heterogeneity of genetical causes of PA seems to be a major hurdle in understanding the PA formation in greater detail. This issue has been overcome in the study of early-onset pituitary gigantism where the impact of microduplication of Xq26.3 encompassing *GPR101* (gene was also found to harbor mutation in 11 sporadic acromegaly patients in the same study) was detected in 14 of 43 study participants demonstrating value of using phenotypic group as uniform as possible (20). Same conclusion can be made to discover that somatic mutations in *USP8* cause corticotroph Pas (42, 43).

The main discovery in our study is the association between the nonsynonymous *MEN1* SNP rs2959656 and the development of PA. Mutations in this tumor-suppressor gene are known to cause sporadic PA and familial MEN1 syndrome (17, 44, 45); however, this particular SNP has not been described previously as a PA marker in the literature. According to the NCBI SNP database, rs2959656 is rare among the CEU population from the HapMap project (34), but the global minor allele frequency is 0.261. It is unlikely that this SNP is the causal variant itself, given the high minor allele frequency in non-European populations. Most likely, this variant is in strong LD with the true causal variant, unique to Europeans or rare in other populations. These assumptions derive from previously published studies, indicating that none of the handful of known *MEN1* mutations is predominantly the cause of neuroendocrine tumors. To date, more than 1300 mutations are known, although there are no mutation hotspots, and functional regions of *MEN1* have not been deduced from these genotype alterations (46, 47).

There were seven rs2959656 in *MEN1* carriers among 143 PA cases and only one carrier in the 354 age- and sex-matched controls. All carriers were heterozygous and all were women. No distinct hormone-secreting profile was observed for carriers. The only minor allele carrier in controls was a 26-year-old female without a pituitary MRI. Her age (26 years) is well below mean age at PA diagnosis (44.3 years) in our sample. Literature data also suggest that pituitary tumors are more likely to affect females and with significantly higher tumor lifetime risk when *MEN1* is mutated (starting from 6.7% at the age of 20–53.4% at the age of 60) (48). This gender discrepancy has been attributed to a difference in the transcriptional regulation of the estrogen and androgen receptors (49). Often, LOH has been observed in patients with MEN1 syndrome (50). LOH in this locus can affect both *MEN1* and *AIP* or either one of them, and such situations have also been associated with Pas (51). Same could be true with inherited large deletion in this region, but not in this case, where all eight carriers were confirmed to be heterozygous for this SNP.

Several associations between SNPs and different PA types, depending on the hormone secretion profile, were observed. The strongest was a previously reported association (28) of rs34037914 in *SSTR5* with acromegaly. A similar association was observed for rs169068 (*SSTR5*), which is in LD with rs34037914. The OR in this study is within the 95% CI range of the previous study (2.63 (1.36–5.08) vs 4.51 (1.76–11.6)) (28). Nevertheless, in this study, we used a larger number of genotype markers resulting in a nonsignificant P-value after correction for 62 SNPs tested. Association of rs34037914 with age at diagnosis of acromegaly in patients with somatotroph adenoma also remained consistent with previous report. Two other markers, rs624975 in *MEN1* and rs1800497 in *DRD2*, showed similar effects as rs34037914 on younger age of the patient at acromegaly diagnosis, while the previously discussed rare rs607969 variant in *MEN1* had a more than two times higher impact on the age at diagnosis. Patients carrying this variant were younger at the time of diagnosis.

Extrasellar growth of adenomas refers to the state when the tumor enlarges to the degree that it extends outside the bony cavity (*sella turcica*) where the pituitary normally resides. Extrasellar growth may be infrasellar (into the bone), suprasellar (toward the optical chiasma and hypothalamus), or into the cavernous sinus. Not surprisingly, most (77% in our sample) of the NFPA cases

exhibited extrasellar growth at the time of diagnosis, because the physical tumor size that usually causes health issues leads to the detection of the disease. Meanwhile, in both hormonally active adenoma groups, where seeking medical help is primarily initiated due to issues linked with hormone oversecretion, the extrasellar growth rate was 46% and was identical in both groups. Due to this heterogeneity, association of extrasellar growth with genotype was carried out only in hormonally active PAs. It is still unclear why several *DRD2* and *GNAS* SNPs might be associated with extrasellar growth of adenomas. First, it is possible that these SNPs increase the aggressiveness of the tumor due to decreased *DRD2* or $G_s\alpha$ content, leading to either less-efficient receptor signaling or signal transduction, resulting in reduced suppression of PA proliferation. In order to test this hypothesis, several consecutive MRI measurements would be needed to assess adenoma dynamics. A second possibility is that these SNPs in *DRD2* and *GNAS* lead to lower hormone secretion; therefore, the adenoma has more time to grow before a person seeks medical help. Third, a completely different mechanism may involve changes in social behavior in the case of *DRD2*. Altered DRD2 signaling may lead to higher anxiety against visiting a medical center and undergoing tests, thus giving more time for tumor growth before its detection. From eight initial associations with the extrasellar growth of PAs, rs7131056, located in the first intron of the *DRD2*, remained significant after correction for covariates and multiple testing for 62 SNPs. According to previous studies, this SNP is weakly associated with migraine (52, 53), nicotine dependence (54), and social phobia (55). The latter association could support the hypothesis that people are more likely to postpone medical examination, allowing their adenoma excess time to grow.

An important limitation of this study is that patient recruitment for the study of acromegaly from two hospitals started earlier than for the rest of the PA cases, leading to overrepresentation of somatotroph adenomas in our sample, which does not reflect the typical proportion of adenoma types in the general population. Other research has shown that the most common type of adenomas is prolactin-secreting (40%), NFPAs (37%), and somatotropinomas (13%) (6). One of the main limitations of this research, and of rare disease genetics in general, is a small sample size that only allows reliable identification of genetic effects with large impacts (OR>3.6 when the allele frequency is 1%). Another limitation of our study is that the control sample had not undergone MRI diagnostics for the pituitary and therefore might contain undiagnosed PAs, especially ones with clinically silent NFPAs. However, cases with silent adenomas that become clinically relevant are generally rare and thus would not affect the analysis significantly. Finally, it should be noted that candidate genes were selected on the basis of their ability to initiate the development of clinically significant tumors rather than tumorigenesis of PA in general.

In conclusion, we have, for the first time, shown an association between rs2959656, a nonsynonymous *MEN1* SNP, with increased risk for PA development. We have also identified several other SNPs in *SSTR5*, *DRD2*, and *GNAS* that show an association trend with the occurrence of PA and their clinical characteristics, but the effects are too small to be reliably proven with our sample set. This study remains one of the few genetic studies using the representative selection of tag SNPs from several candidate genes in order to identify genetic causes of PA.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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3.4 Identification of Somatostatin Receptor Type 5 (SSTR5) gene polymorphisms associated with acromegaly

The author of the thesis is fourth listed author in the 2009 publication "Identification of Somatostatin Receptor Type 5 (SSTR5) gene polymorphisms associated with acromegaly", where he performed independent checking of results and supporting data tables, edition of the manuscript, implementing changes and clarifications as requested by the referees.

CLINICAL STUDY

Identification of somatostatin receptor type 5 gene polymorphisms associated with acromegaly

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Abstract

Objective: The aim of this study was to characterize the genetic variance of somatostatin receptor 5 (SSTR5) and investigate the possible correlation of such variants with acromegaly risk and different disease characteristics.
Design and methods: The SSTR5 gene coding region and 2000 bp upstream region was sequenced in 48 patients with acromegaly and 96 control subjects. Further, three single nucleotide polymorphisms (SNPs) were analyzed in the same group of acromegaly patients and in an additional group of 475 age- and sex-matched controls.
Results: In total, 19 SNPs were identified in the SSTR5 gene locus by direct sequencing. Three SNPs (rs34037914, rs169068, and rs642249) were significantly associated with the presence of acromegaly using the initial controls. The allele frequencies were significantly ($P<0.01$) different between the acromegaly patients and the additional large control group. rs34037914 and rs642249 remained significantly associated with acromegaly after Bonferroni correction and permutation tests (odds ratio (OR)=3.38; 95% confidence interval (CI), 1.78–6.42; $P=0.00016$ and OR=2.41; 95% CI, 1.41–4.13; $P=0.0014$ respectively). Haplotype reconstruction revealed two possible risk haplotypes determined by rs34037914 (633T) and rs642249 (1044A) alleles. Both haplotypes were found in significantly higher frequency in acromegaly patients compared with controls ($P<0.001$). In addition, the 663T allele was significantly associated with a younger age of acromegaly diagnosis (unstandardized regression coefficient $\beta=-10.4$; $P=0.002$), increased body mass index ($\beta=4.1$; $P=0.004$), higher number of adenoma resection ($P<0.001$) and lack of observable tumor shrinkage after somatostatin analog treatment ($P=0.014$).
Conclusions: Our results demonstrate a previously undetected strong association of two SSTR5 SNPs with acromegaly. The data also suggest a possible involvement of SSTR5 variants in decreased suppression of GH production and increased tumor proliferation.

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Introduction

Acromegaly is a rare chronic disease mainly caused by pituitary GH overexpressing adenomas (1). The general characteristics of acromegaly are elevated levels of GH and insulin-like growth factor 1 (IGF1). Disease incites high morbidity and increased risk of mortality if it is not properly treated (2). One of the main physiological mediators of GH secretion and regulation of tumor proliferation is somatostatin acting through the somatostatin receptors (SSTRs), a family of G protein-coupled receptors (GPCRs) including five subtypes (SSTR1–5) (3, 4). Two of these receptor subtypes SSTR2 and SSTR5 are found in high levels in pituitary somatotropinomas (5), which is in contrast to other SSTR subtypes. Accordingly, somatostatin analogs (SA) such as octreotide and lanreotide, that have the highest affinity to SSTR2 and SSTR5, are used to repress GH secretion in acromegaly patients (6, 7). Although the introduction of these drugs has greatly improved therapy for the disease, a significant proportion of the patients do not adequately respond to treatment resulting in incomplete reduction in GH and IGF1 levels (8, 9). Moreover, 10% of the acromegaly patients show no changes in GH levels upon the SA treatment (10).

The genetic basis of acromegaly and drug resistance remains largely unclear (reviewed in (11)). It has been shown that somatic mutations in the Gsα gene (*GNAS*)

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are associated with constitutive activity of adenylyl cyclase and that these mutations are found in roughly 40% of somatotropinomas resulting in increased sensitivity to the inhibitory action of somatostatin (12). Very few germline mutations are known that are implicated in inherited pituitary tumor risk. Recently, it was shown that pituitary adenoma predisposition in familial cases are due to mutations in the aryl hydrocarbon receptor-interacting protein (*AIP*) gene (13) and may also play a role in aggressive disease development in sporadic cases of somatotropinomas (14, 15). As far as the acromegaly and SSTRs are concerned, only a few genetic association studies have been conducted. Filopanti *et al.* (16) identified that the rs3751830 and rs169068 alleles are weakly associated with altered GH and IGF1 levels in patients with acromegaly. To date, the only non-synonymous SSTR mutation that may be associated with drug resistance in acromegaly treatment is a germline R240W mutation in the SSTR5 that was found in one patient resistant to octreotide (17). Since almost no genetic mutations of SSTR2 and SSTR5 have been found in resistant patients (18, 19), the resistance has been attributed to impaired SSTR2 and SSTR5 expression in tumor tissues (10, 20).

In this study, we performed sequencing of the SSTR5 gene in 144 subjects and investigated potential correlations between SSTR5 gene variants and disease outcome as well as the clinical and hormonal characteristics in 48 acromegaly patients and 475 controls.

Materials and methods

Study group

We followed the STREGA guidelines (21) to describe the study group selection and association analysis. Case–control study groups were selected from the Latvian Genome Database (LGDB), a government funded biobank. All participants of LGDB were over 18-years of age, and information about their health status was affirmed by physicians using International Classification of Diseases (ICD)-10 codes. Anthropometric measurements (including weight and stature) were obtained by direct measurement; ethnic, social, environmental information, and familial health status were obtained using a questionnaire-based interview. Participants of the LGDB were recruited by medical personnel in hospitals or general practices. Recruitment was population based (a specific health condition was not the obligatory requirement for involvement). Signed consent forms were acquired from all participants. The Biobank protocol was approved by the Central Medical Ethics Committee of Latvia (protocols no. A-30, 2005 and A-7, 2007).

Forty-eight acromegaly patients recruited for this study were enrolled on to the LGDB between 2004 and 2008 from two main hospitals: Pauls Stradins Clinical University Hospital (41 patients) and Riga Eastern Clinical University Hospital (seven patients) representing ~80% of all the acromegaly patients registered in Latvia as recorded in October 2008 (diagnosed with acromegaly from 1985 to 2007). Additional data were collected based on hospital records and interviews for all the patients selected for the study (ICD-10 code E22.0). Forty-five patients received the SA octreotide (Sandostatin LAR) at dose 10–30 mg in every 28 days or lanreotide (Somatuline Autogel) at dose 60–90 mg in every 28 days. Tumor size was measured as the maximum diameter obtained from magnetic resonance imaging data and tumors were classified accordingly as microadenomas (<10 mm) or macroadenomas (≥10 mm). The effect of SA on tumor proliferation and IGF1 level normalization was estimated by comparing the tumor sizes and serum IGF1 (μg/ml) measurements during the course of therapy. To estimate the dynamics of adenoma size, the data from last follow-up was compared with the first available measurement with at least a 12-month period in between. We excluded all cases where the therapy was interrupted or adenoma resections were performed during this period. Two groups were defined: 'reduced' with observable tumor shrinkage ($n=11$) and 'unchanged' with no observable tumor shrinkage ($n=22$) together with the cases that showed prolonged expansion ($n=2$). Only a limited number of cases had IGF1 measurements available before the therapy. For the IGF1 response, only the data at least 6 months after the start of the SA therapy were considered for analysis and only if the therapy was not interrupted. In those cases where several IGF1 measurements were available, the mean IGF1 was calculated, excluding the outliers where possible. Non-responsiveness was defined as the mean IFG1 value above the upper limit of normal (ULN) value at the corresponding age. Owing to the lack of uniformity in the GH measurements, we did not include the GH levels in this analysis.

As controls (control I), 96 samples were randomly chosen for sequencing from LGDB participants excluding patients with metabolic and endocrine diseases. To minimize the risk of false positivity, we selected an additional control group (control II) who were sex- and age-matched consisting of 475 LGDB participants. As per the selection criteria, we used 63 participants from the control I who were also included in the control II group. Detailed sample selection procedure is described in Supplementary Material, see section on supplementary data given at the end of this article. Study protocol was approved by Central Medical Ethics Committee of Latvia (protocols no. A-33, 2005 and A-3, 2008).

DNA analysis

DNA samples were provided by LGDB and aliquoted into 96-well PCR plates or PCR tubes by Tecan Freedom Evo robotic pipette. The final DNA amount was 28 ng/well.

The SSTR5 gene containing genomic DNA region including 5′ and entire coding region (from −2239 to +1294 respective to start codon) were amplified in six PCR reactions and sequenced using BigDye chemistry and ABI Prism 3100 (AME Bioscience, Toroed, Norway) capillary electrophoresis sequencer. All chromatograms were manually inspected using Contig Express Software of Vector NTI Advance 9.0 package (Invitrogen). Presence of polymorphisms was confirmed by opposite strand analysis. Genotyping was carried out using minisequencing and subsequent MALDI-TOF mass spectrometry analysis. Primers were designed on Primer3 Software (source code available at http://fokker.wi.mit.edu/primer3/) and using the program CalcDalton (www.uni-leipzig.de/~ahnert/calcdalton.htm). Detailed experimental procedures can be found in Supplementary Material and primer sequences can be found in Supplementary Table 1, see section on supplementary data given at the end of this article.

Statistical analysis

Statistical analysis was performed by the PLINK 1.07 (22) and SPSS (standard version 13; SPSS, Chicago, IL, USA) software. Deviation from Hardy–Weinberg equilibrium was assessed by the exact test described by Wigginton *et al.* (23) which is considered more accurate for rare genotypes. The Cochran–Armitage trend test was used for association analysis in the case–control group and Bonferroni correction was applied. Haplotype association was performed as implemented in PLINK. For the quantitative analyses, the IGF1 data were transformed as a normalized percentage of ULN of appropriate age according to formula $(C_{IGF1}-ULN_{IGF1})/ULN_{IGF1}\times 100)$. Normalized IGF and all other continuous variables displayed normal distribution and were further used in linear regression analysis. Two-sided Fisher exact test was used to test the allelic distribution in the case of categorical clinical variables, except the analysis of number of adenoma resections where Pearson χ^2 was calculated from 3×3 table. Permutation tests with 100 000 permutations were performed for each analysis and we used corrected (EMP2) *P* values. These values are corrected based on calculation of the proportion of permutations in which any of the test statistics exceeds the particular observed statistic and are more stringent than uncorrected *P* values.

Results

DNA samples of all 48 available acromegaly patients and 96 control individuals were subjected to direct sequencing of the SSTR5 gene including both the coding and the flanking regions (−2239 to 1294 respective to start codon). Baseline demographic and clinical characteristics are given in Table 1. In total, 19 polymorphisms were identified (relative positions, sequencing success rate and Hardy–Weinberg test results are shown in Supplementary Table 2, see section on supplementary data given at the end of this article). All single nucleotide polymorphisms (SNPs) were in Hardy–Weinberg equilibrium. Fourteen SNPs passed the quality and minimal minor allele frequency (MAF) criteria and were included in the subsequent analysis. Three SNPs were excluded due to low sequencing success rate (<95%) and two SNPs were excluded due to low MAF (<0.01).

Table 1 Characteristics of the study population. Data are presented as mean (S.D.) or *n* (%).

Variables	Acromegaly patients (*n*=48)	Control I (*n*=96)	Control II (*n*=475)
Sex			
Female	32 (67%)	67 (70%)	324 (68%)
Male	16 (33%)	29 (30%)	151 (32%)
Age (years)	55.8 (12.8)	53.2 (17.4)	54.3 (13.4)
BMI (kg/m²)	29.9 (5.5)	25.4 (4.4)	27.6 (5.2)
Waist (cm)	91.0 (13.7)	–	–
Age at diagnosis (years)	47.4 (13.1)	–	–
Tumour size			
Macroadenoma	31 (64%)	–	–
Microadenoma	17 (36%)		
Effect of SA on tumor size (*n*=35)			
Reduced	11 (31.5%)	–	–
Unchanged	24 (68.5%)		
Expanded			
Adenoma resections per patient			
1	23 (47.9%)	–	–
2	2 (4.1%)		
3	2 (4.1%)		
IGF1 norm			
% ULN before treatment (*n*=20)	178.8 (87.9)	–	–
% ULN after treatment (*n*=39)	50.8 (77.8)		
IGF1 responsiveness (*n*=39)			
<ULN	12 (30%)		
>ULN	27 (70%)		

BMI, body mass index; IGF1, insulin-like growth factor 1; ULN, upper limit of normal at corresponding age group; IGF1 norm, % ULN, normalized percentage of ULN $((C_{IGF1}-ULN_{IGF1})/ULN_{IGF1}\times 100))$.

The minor alleles of three SNPs were independently associated with the presence of acromegaly (Table 2) using Cochran–Armitage trend test. T and A alleles of rs34037914 and rs642249, respectively, remained associated with acromegaly after adjusting for multiple comparisons using both Bonferroni correction and adjusted permutation test. The first control group (control I) contained some individuals with malignant and benign neoplasms (23%). To avoid the influence of age, gender, or medical conditions on the association results, as well as to minimize type I error, we selected an additional age- and gender-matched control group representing 475 healthy individuals. Genotyping of the three SNPs (rs34037914, rs169068, and rs642249) associated with acromegaly was performed in this study group. Genotyping results for these individuals corresponded to the genotypes previously obtained from the

Table 2 Single nucleotide polymorphism (SNP) association analysis in acromegaly patients and controls.

SNP codes	Alleles/ position[a]	AA change	MAF case	MAF controls	Allelic OR (95% CI)	P trend[b]	P perm[c]
Acromegaly patients (n=48) versus control I sample (n=96)							
rs550713	T-2190G		0.078	0.036	2.23 (0.76–6.56)	0.16	0.84
NA	T-2138delT		0.427	0.479	0.81 (0.49–1.34)	0.43	1
rs535338	A-1670G		0.202	0.266	0.7 (0.38–1.27)	0.24	0.97
NA	C-805G		0.043	0.044	0.97 (0.28–3.3)	0.96	1
rs4988479	G27A		0.021	0.031	0.67 (0.13–3.4)	0.63	1
rs4988483	C142A	P335L	0.021	0.016	1.34 (0.22–8.16)	0.75	1
rs4988484	C155T	A52V	0.021	0.042	0.49 (0.1–2.35)	0.40	1
rs4988487	C325T	P109S	0.021	0.042	0.49 (0.1–2.35)	0.40	1
rs35072648	G516A		0.031	0.047	0.66 (0.17–2.48)	0.56	1
rs34947461	G573A		0.021	0.031	0.66 (0.13–3.33)	0.65	1
rs34037914	**C633T**		**0.146**	**0.036**	**4.51 (1.76–11.6)**	**0.0015***	**0.019**
NA	G693A		0.021	0.016	1.34 (0.22–8.16)	0.75	1
rs169068	**T1004C**	**P335L**	**0.594**	**0.429**	**1.94 (1.18–3.21)**	**0.013**	**0.11**
rs642249	**G1044A**		**0.208**	**0.073**	**3.35 (1.61–6.97)**	**0.0016***	**0.020**
Acromegaly patients (n=48) versus Control II sample (n=475)							
rs34037914	**C633T**		**0.146**	**0.048**	**3.38 (1.78–6.42)**	**0.00016***	**0.0012**
rs169068	**T1004C**	**P335L**	**0.594**	**0.434**	**1.91 (1.25–2.93)**	**0.0029**	**0.0089**
rs642249	**G1044A**		**0.208**	**0.098**	**2.41 (1.41–4.13)**	**0.0014***	**0.0064**

AA, amino acid; MAF, minor allele frequency; OR, odds ratio; CI, confidence interval; NA, not acquired; association results for SNPs with $P<0.05$ are marked with bold. *Significant after Bonferroni correction (significances level 0.0029).
[a]Nucleotide position relative to the SSTR5 start codons, major allele is shown before and minor allele after the number indicating the nucleotide position.
[b]P value from Cochran–Armitage trend test.
[c]P value obtained from 100 000 permutation and corrected for multiple testing (EMP2).

sequencing reactions. All three SNPs were significantly associated with the presence of acromegaly (Table 2).

Six haplotypes were derived from the three candidate SNPs in acromegaly patients and control II group (Table 3). Four haplotypes (frequency >1% in control group) were subjected to the haplotype-based association analysis. The overall joint test of the SSTR5 haplotypes on presence of acromegaly (comparing acromegaly patients and control II group) was highly significant ($P=3.4\times10^{-6}$). For haplotype-specific tests the most common haplotype (56%) that contains only the common alleles of the three included SNPs (C-T-G) was chosen as a reference haplotype (Hap1). Hap3 (C-C-A) and Hap4 (T-C-G) contained the independently associated alleles of rs642249 and rs34037914, respectively, and were found with significantly higher frequency in acromegaly patients than in controls. Both these haplotypes also contain the C allele of rs169068 that was independently associated with acromegaly. On the contrary, Hap2 (C-C-G), which in addition to common rs642249 and rs34037914 alleles contains the rs169068 C allele, was not associated with acromegaly indicating that the association of this allele with the acromegaly is due to linkage disequilibrium (LD) with the minor alleles of both other SNPs.

We also tested the association of rs642249 and rs34037914 with different disease characteristics and phenotypes affected by acromegaly (Tables 4 and 5). The following categorical variables in the group of acromegaly patients were tested: size of adenoma (microadenoma versus macroadenoma), change in the size of adenoma as a result of use of medication (reduced versus unchanged and increased); number of adenoma resections per patient (0 vs 1 vs 2–3); and IGF1 levels

Table 3 Haplotype based association in acromegaly patients and control II sample.

Names	Haplotypes[a]	Haplotype frequency (number)		OR (95% CI)	P values[b]
		Cases	Control II		
Hap1	C-T-G	0.385 (37)	0.562 (534)	Ref	Ref
Hap2	C-C-G	0.260 (25)	0.293 (279)	1.3 (0.76–2.2)	0.3358
Hap3	C-C-A	0.188 (18)	0.096 (91)	**2.85 (1.56–5.22)**	**0.0016**
Hap4	T-C-G	0.146 (14)	0.045 (43)	**4.69 (2.35–9.34)**	**0.000053**
Hap5	C-T-A	0.021 (2)	0.002 (2)	NC	NC
Hap6	T-T-G	0 (0)	0.001 (1)	NC	NC

OR, odds ratio; CI, confidence interval; Ref, reference group; NC, not calculated; association results for haplotypes with $P<0.05$ are marked with bold.
[a]SNP order=rs34037914 – rs169068 – rs642249.
[b]P value from Fisher exact test.

Table 4 Categorical analysis of SNP association with different phenotypes in acromegaly patients.

Phenotype	Genotype distribution	MAF	Allelic OR (0.95% CI)	*P* value[a]	*P* perm[b]
SNP: rs34037914					
TT/TC/CC					
Size of adenoma			1.44 (0.42–5.00)	0.56	0.62
Micro	0/4/13	0.11			
Macro	2/6/23	0.16			
Effect of SA on tumor size			NA	**0.014**	**0.02**
Reduced	0/0/11	0			
Enlarged	2/7/15	0.22			
IGF1 after treatment			3.27 (0.64–16.54)	0.13	0.17
Below ULN	0/2/11	0.07			
Above ULN	2/5/14	0.21			
Number of adenoma resections		NC	NA	**0.0001**[c]	NC
0	0/4/16				
1	0/5/18				
2–3	2/1/1				
SNP: rs642249					
AA/AG/GG					
Size of adenoma			1.03 (0.28–3.82)	0.63	0.68
Micro	2/4/11	0.19			
Macro	2/8/21	0.24			
Effect of SA on tumor size			0.78 (0.28–2.15)	0.96	0.96
Reduced	1/2/8	0.19			
Enlarged	2/5/17	0.18			
IGF1 after treatment			1.04 (0.33–3.31)	0.94	0.95
Below ULN	2/2/9				
Above ULN	1/8/12	0.24			
Number of adenoma resections		NC	NC	0.5[c]	NC
0	2/6/12				
1	2/6/15				
2–3	0/0/4				

OR, odds ratio; CI, confidence interval; MAF, minor allele frequency; NA, not applicable; NC, not calculated. Association results with $P<0.05$ are marked in bold.
[a] *P* value from Fisher exact test.
[b] *P* value obtained from 100 000 permutation.
[c] *P* value from Pearson χ^2 calculation using 3×3 table (4 degrees of freedom).

after medical treatment (normal versus increased). Body mass index (BMI), age at acromegaly diagnosis and age-adjusted normalized IGF1 levels were used as continuous variables in the linear regression analysis (Table 5). rs642249 had no association with any of these phenotypes. However, rs34037914 was significantly associated with the number of these phenotypes (summarized in Supplementary Figure 1, see section on supplementary data given at the end of this article). Carriers of the rs34037914 T allele were characterized by significantly lower mean age at diagnosis (*P* value=0.002) and increased BMI value (*P* value=0.004). Association of the T allele with these variables corresponded best to the additive genetic model (Tables 4 and 5, Supplementary Figure 1, see section on supplementary data given at the end of this article). No association ($P=0.56$) was observed between rs34037914 and increased waist circumference (this measurement was only available for 23 patients) indicating that association of rs34037914 with increased BMI in this group is due to an increase in muscle and bone mass caused by acromegaly rather than to an increase in fat mass. In addition, we tested whether the rs34037914 allele is associated with BMI and stature in the healthy individuals, but we found no differences between the genotype groups (data not shown) for any of the genetic models. Interestingly, the rs34037914 risk (T) allele was absent in the group of patients with observable tumor shrinkage as a result of octreotide or lanreotide treatment, resulting in association between this parameter and presence of the rs34037914 T allele (*P* value=0.014). Even more intriguing, the presence of the same allele was positively correlated with the number of adenoma resections per patient (*P* value=0.0001). Among the four patients in the entire study group who had more than one adenoma resection, two were homozygotes for the rs34037914 T allele and one was a carrier of the same allele in the heterozygous state. We also observed an increased rs34037914 T allele frequency in patients who failed to normalize their IGF1 levels compared with those who reached normal IGF1 levels after the SA treatment. Similarly, the mean normalized IGF1 levels were increased in patients with rs34037914 CT and TT genotypes in an additive manner (Table 5). None of these differences, however, reached a statistical significance.

Table 5 Qualitative analysis of SNP association with different phenotypes in acromegaly patients.

	Phenotypes (mean ± S.E.M.)		
Genotype	BMI (kg/m^2)	Age at diagnosis (y)	IGF1 % ULN
SNP: rs34037914			
CC	**28.87 ± 0.80**	**50.43 ± 2.03**	45.40 ± 14.61
CT	**31.35 ± 2.00**	**40.60 ± 3.90**	63.23 ± 30.03
TT	**40.48 ± 1.03**	**28.50 ± 7.50**	80.23 ± 32.28
P value[a]	**0.004**	**0.002**	0.41
P perm[b]	**0.012**	**0.006**	0.81
SNP: rs642249			
GG	31.04 ± 1.06	27.19 ± 1.29	28.33 ± 0.98
GA	48.25 ± 2.33	46.00 ± 3.68	44.50 ± 8.85
AA	40.72 ± 16.08	88.52 ± 19.92	19.93 ± 23.53
P value[a]	0.09	0.51	0.23
P perm[b]	0.23	0.87	0.97

IGF1 % ULN, normalized percentage of ULN $((C_{IGF1} - ULN_{IGF1})/ULN_{IGF1} \times 100)$. Association results with $P<0.05$ are marked in bold.
[a]*P* value from linear regression.
[b]*P* value obtained from 100 000 permutation.

Discussion

In this study, we present a novel and highly significant association of two SNPs, rs34037914 and rs642249, in the SSTR5 gene with acromegaly. This association remains significant after both Bonferroni correction and adjusted permutation tests. Both SSTR2 and SSTR5 are potential candidate genes for an increased risk to develop acromegaly and poor response to SA, due to their important role in controlling GH secretion and somatotroph growth. However, until now there has been a lack of convincing association between genetic variants of any of the SSTRs and acromegaly or resistance to the action of somatostatin or SA (reviewed in (11)). This can be explained in part by the observation that rare SNPs are often not included in genotyping due to low power in studies with a limited number of patients. Thus, to avoid a selection bias, an important issue in genetic studies of rare disease, we performed sequencing of the SSTR5 gene in all available patients and substantial number of controls. We identified four novel polymorphisms (one single nucleotide deletion and three SNPs) that were not previously reported to the SNP databases among the total of 19 polymorphisms (Supplementary Table 2, see section on supplementary data given at the end of this article). The allele frequencies of the previously known SNPs showed a variable prevalence, which was comparable to the allele frequencies reported in databases or previously published reports (http://www.genecards.org/cgi-bin/carddisp.pl?gene=SSTR5&snp=207&search=sstr%205&rf=/home/genecards/current/website/carddisp.pl#snp). The prevalence of three SNPs was significantly different in acromegaly patients compared individually with a sex- and age-matched control group. Our haplotype analysis suggests that the non-synonymous polymorphism rs169068 (Pro335Leu) is not associated with acromegaly. The difference in allele frequencies between cases and controls for this SNP can be explained by strong LD between rs169068 C allele (Pro) and both associated alleles of rs642249 and rs34037914. Interestingly, the C allele of rs169068 has been previously associated with decreased IGF1 (16) in acromegaly patients. Our results may rather suggest an opposite effect due to LD between the C allele and rs34037914 T allele, as the latter has a non-significant tendency toward increased IGF1 levels. Unfortunately, the rs34037914 and rs642249 were not genotyped in a study performed Filopanty *et al.* (16) The C allele of the rs169068 was associated with the presence of bipolar affective disorder in a British but not in a Danish population (24), while there was a lack of association with autism in both Danish and French populations (25). No association was found between this SNP and risk or survival of pancreatic cancer (26). To our best knowledge, the rs34037914 and rs642249 have not been tested previously with respect to acromegaly. The presence of the A allele of rs642249 was associated with increased mean IGF1 levels, but not with risk of breast cancer in the international EPIC study (27). Neither rs34037914 nor rs642249 has shown associations with bipolar affective disorder (24).

The importance of the four SNPs from the SSTR5 gene locus on regulation of IGF1 levels has recently been demonstrated in a large multinational study on samples from the Breast and Prostate Cancer Cohort Consortium (28). One of these SNPs, the rs3751830, was also found in our study, but was not included in the final analysis due to low genotype call rate (Supplementary Table 2, see section on supplementary data given at the end of this article). However, no significant differences were found between the allele frequencies of this SNP and any of the phenotypes studied (data not shown). Data on LD between associated SNPs rs34037914 or rs642249 and other SNPs from the above mentioned study are not available and it is difficult to assess if these SNP may be part of the same haplotype or if they could represent functional SNPs. It is also clear that physiological consequences of the same genetic SSTR5 variants may be very different when a normal pituitary gland is considered compared with pituitary tumors. Unfortunately, the IGF1 levels for the control group were not available in our study, and this would help to assess the effects of these SNPs in on IGF1 regulation in healthy individuals.

rs34037914 shows the strongest association with acromegaly (odds ratio = 3.38, 95% confidence interval = 1.78–6.42) assuming an additive mode of inheritance. We strongly suggest that this is not a false-positive finding since the same rs34037914 allele is associated with a number of independent clinical characteristics in our group of acromegaly patients. This is the first study that reports such a pronounced effect of genetic variants on body mass in the case of

somatotropinomas. The median BMI was 12 kg/m^2 larger in the T allele homozygotes compared with wild-type (wt) homozygotes. Similarly, the median age at diagnosis was 22 years earlier considering the same group of patients. This is in line with previous data obtained in a large international collaborative study showing earlier onset age when investigating germ line mutations in the AIP (15) a gene that initially was associated with familial pituitary adenoma cases (13). According to our data, the T allele of rs34037914 predisposes to increased aggressiveness and post-surgical reoccurrence of pituitary tumors as well as non-responsiveness to antiproliferative effects of SA. The increased BMI in carriers of the T allele, however, indicates that the effect of this polymorphism is not limited to the regulation of tumor cell proliferation, but also affects the systemic GH levels. We did not observe the same effects of rs642249, the other acromegaly associated SNP, on the clinical or hormonal characteristics in our patient group. If not false positive, this may be explained by the smaller effect size of this SNP and a larger cohort of acromegaly patients would be needed to provide conclusive results. Alternatively, the disease predisposing effect linked to rs642249 may not be associated with characteristics available in our study.

It is not clear how the synonymous rs34037914 is connected with functional effects. First, other functional mutations in LD with rs34037914 may exist outside the regions we sequenced. It is plausible that such mutations, or rs34037914, are influencing the expression of SSTR5. Although the unresponsiveness to SA has been clearly associated with low expression of SSTR2 (29–31), the SSTR5 is the most abundantly expressed receptor in somatotropinomas (18, 32, 33) and it plays an important role in mediating the effect of somatostatin. The relative contribution of each of those receptors in the control of GH secretion is still unclear. It is established that both receptors are needed for hormonal regulation since the activation of SSTR2 and SSTR5 results in a synergistic effect on GH release (34–36). Thus, if the rs34037914 or rs642249 is linked to the changes in SSTR5 expression, they may significantly influence the responsiveness of the pituitary tumor cells to somatostatin and its analogs. Genotyping the rs34037914 and rs642249 in patients with known SSTR5 and SSTR2 expression profiles from pituitary tumors would help to test this hypothesis. Another possibility is that the particular sequence variations may induce alternative/*de novo* splicing creating a non-functional receptor protein or receptor with altered functions. It has indeed been shown that SSTR5 is found in two isoforms in pituitary tumors and that these are presumably generated by a splicing of the SSTR5 involving the presence of a cryptic donor and acceptor splice site (37). One of these variants, named SST5TMD4, has been found to be abundantly present in octreotide-resistant somatotropinomas and could interfere with the normal inhibitory response of adenomas to somatostatin (38). rs34037914 is actually located close to the splice site of the other variant SST5TMD5, and it is possible that this polymorphism may be important for this donor splice site formation. Functional consequences of the truncated receptor protein could involve its ability to interact with SSTR5 or SSTR2 leading to non-functional heterodimers. It has been shown previously that SSTR5 can form heterodimers with different GPCRs (reviewed in (39)), including SSTR2 and dopamine D2 receptors heterodimers with enhanced functionality (40, 41).

It is unlikely that changes in the amino acid sequence are responsible for the effects found in our study considering the functional importance of these SNPs. The non-synonymous substitution rs169068 is located in the C-terminal intracellular tail of the receptor causing a proline to leucine change. It has been shown by mutational analysis that the C-terminal domain is involved in the interaction with adenylate cyclase and is important for desensitization and internalization of this receptor (42). A recent study has shown that SSTR5 with leucine at position 335 loses its inhibitory effect on cell proliferation compared with the proline variant (43). However, It is unlikely that this substitution has a major impact on tumor development or drug resistance in our study group, as the proline was actually more frequent among the acromegaly patients (59.4% C allele frequency) while a majority of the general population carry SSTR5 with leucine in this position (56.6% T allele frequency). According to the SNP NCBI database and the HapMap data on European, African, and Asiatic populations (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=169068), the distribution of this variant differs significantly among the ethnic groups. The frequency of the T allele, as far as the HapMap data is considered, varies from 27% in the African American population and 52% in the Caucasian population to 85% in the Japanese and Chinese populations.

The major limitation of this study is the lack of standardized GH and IGF1 measurements for all acromegaly patients that did not allow a full estimation of the influence of these polymorphisms on hormonal regulation and drug responsiveness. Similarly, it was not possible to test the eventual effect of polymorphisms on the level of SSTR5 expression due to the unavailability of the tumor tissue samples. Replication studies with a larger patient size and other ethnic groups would provide additional insight into the associations that we have identified.

In conclusion, we have identified genetic variations in SSTR5 that are strongly associated with acromegaly and several of the clinical characteristics related to this disease. If tested functionally and proven clinically rs34037914 has a potential to become a diagnostic marker of non-invasive tests to determine the prognosis and aggressiveness of somatotropinomas.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/EJE-11-0416.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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4. Discussion

Multifactorial or non-communicable diseases, which are contributing to a severe increase in human morbidity and mortality, are on the rise throughout the world and they are burdensome to national healthcare systems.

Uncovering the genetics of multifactorial diseases may help to understand their molecular mechanisms which can lead to a targeted search for therapies, precision medicine approaches tailored to the individual, and more effective use of (always) limited healthcare funding.

Three broad classes of multifactorial diseases, which include cardiovascular diseases (especially CAD), diabetes and tumors; most of which are malignant, are the main cause of disability adjusted life years lost worldwide (Murray *et al.* 2012).

These conditions are being observed more frequently due to population aging trends, human population expansion, a greater awareness of these diseases, improved diagnostic capacity, and their share in total disease burden is increasing (Das and Samarasekera 2012, Murray *et al.* 2012).

CAD, diabetes and pituitary adenomas are connected at a physiological level since hormonal imbalances caused by pituitary adenoma can lead to diabetes insipidus. An increase in glucose levels due to diabetes is a stress factor for endothelium cells and is therefore associated with atherosclerosis; an underlying process in CAD. PA includes various cardiovascular conditions. These conditions seem to share the same genetic architecture from a causal perspective and are a heterogeneous group of diseases with shared symptoms and endpoints from a molecular perspective. This makes these disorders prime targets for theranostics (Choi *et al.* 2015, Patel and Janjic 2015). CAD, diabetes and pituitary adenomas are each associated with multiple genetic variations, intermediate phenotypes, and complications. Novel genetic markers are still being found after decades of scientific research (Nikpay *et al.* 2015). Missing heritability and disease mechanisms from established genetic risk factors and associated phenotypes is still not understood in detail. The vast majority of studies on multifactorial diseases have been conducted in Western European or American populations. This has led to many important discoveries but has also introduced population biases. Much information that could consequently have been acquired due to the

scale by which research efforts were launched and financial resources that were invested, has been missed (Kessler *et al.* 2016).

4.1 Adenosine receptor gene variants as possible minor modulators of CAD

Adenosine and its four receptors (A1, A2A, A2B and A3) play a role in cardio protection in ischemia cases. This is achieved by limiting the damage caused by a decrease in, or complete lack of, oxygen due to obstructed blood flow in the coronary arteries of the heart. A large meta-analysis of GWAS studies for the imputation of genetic variants was based on information obtained from the 1000 Genomes Project. The meta-analysis identified variation near the A2A receptor where the common allele G is significantly associated with an increased risk of CAD (OR=1.20, CI 0.95=1.13-1.27, P=1.60×10^{-10}) (Nikpay *et al.* 2015).

The lead SNP rs180803 (Chr22q11.23 *POM121L9P – ADORA2A*) is located in the non-coding RNA POM121 transmembrane nucleoporin-like 9 pseudogene (*POM121L9P*) locus (Yates *et al.* 2016).

A genome-wide significant association (P<5x10-8) was observed for 21 variants in a 1.2Mb region. The majority of these variants are located in introns of *A2A* and *SPECC1L* genes. They are also in high LD (R^2>0.6) with the rs180803 lead variant (Nikpay *et al.* 2015). This adds to the evidence that genetic variations in the adenosine system are involved with cardio protection. Our study published in 2011 was focused on the adenosine A3 receptor located on chromosome 1p13.2, and its associated variant (dbSNP ID rs35511654). Modest protective action of the minor allele C was observed (OR=0.71, CI 0.95=0.53–0.96) (or increased OR for the common allele to 1,41). The variation causes a missense mutation which changes the amino acid of the A3 receptor in 248th position from isoleucine to leucine. Chemically, these amino acids are closely related; the only difference is the branching point of the side chain. Nevertheless, in the case of the A3 receptor and the rs35511654 variant, the position of the variant could crucially influence the binding capacity of the agonist. Functional studies have reported that nearby amino acid positions are important for the recognition of agonists and antagonists (p.250 N, two amino acid residues away and p.247 S, one amino acid residue away). Moreover, the W243 amino acid, which is five positions away from altered position by rs35511654, is implicated in signal transduction while the E239 amino acid (nine positions away from p.248 I) is associated with receptor activation (Gao *et al.* 2002). The various three-dimensional space requirements of different side chains, as coded by rs35511654, could lead to modified spatial distances. This subsequently could lead

to either enhanced recognition of agonists, signal transduction or receptor activation, or decreased recognition of antagonists. This would be in line with the protective nature of the minor allele, as shown in our study. Usually, depending on the cell type and tissue in which it is located, one component in human cells has various roles. This could be the same in the case of A3R in that it could be utilized in protecting the heart and its blood vessels. Alternatively, it could act as a protector from reperfusion injury in a way that is different in immune cells since A3R inhibits the release of TNF-alpha from monocytes; the main drivers of plaque formation in the coronary arteries (Zhang *et al.* 2005). Due to the different regulation in different cell types, the impact of the rs35511654 polymorphism could vary depending on the tissue it is located in.

Nevertheless, our observation has not been confirmed in candidate gene studies or GWAS analyses in any other population. A prominent hotspot for CAD-associated variants was found to be 2.22Mb away in a GWAS study. The rs646776, rs599839, rs12740374, and rs629301 polymorphisms are located in a noncoding DNA region between the *CELSR2* and *PSRC1* genes (Musunuru *et al.* 2010). It was found that this locus is associated with increased LDL cholesterol levels as well as an increased risk of MI and that the risk allele is common one (Samani *et al.* 2007). According to Musunuru *et al*. the mechanism of the lead rs12740374 SNP is an increase in *PSRC1* and *SORT1* expression in the liver and a resulting increase in very small dense LDL particle levels in the blood (Musunuru *et al.* 2010). According to literature, these very small dense LDL particles are more important in promoting atherogenesis than LDL in larger aggregations (Berneis and Krauss 2002). The most interesting finding from our study is that the common allele predisposes individuals to an increased risk of developing CAD. It is possible that the rs35511654 variant from our study serves as a weak proxy to the *PSRC1/SORT1* risk-associated variants. This observation could become more succinct through the genotyping of the 1p13.2 locus to establish if there is linkage disequilibrium between the variants in the Latvian population. The second interesting point is that an additional member of the adenosine receptors, A2A, is situated at a locus where the common allele increases CAD risk (Nikpay *et al.* 2015). These facts allow us to formulate the hypothesis that evolution is selecting for cardio-protecting variants and that their frequency is on the rise in the general population.

The majority of large scale CAD studies have been performed using populations of Western Europe descent (Table 2, Table 3). This choice was logical because of the advancement in science and economic situations in the countries where the studies were conducted. Despite

this, these studies have probably only captured a small part of the genetic variation contributing towards human CAD. Indeed, while trying to narrow down the variation responsible for the increased CAD risk, a study has used African American samples in order to single out the most likely candidate (Musunuru *et al.* 2010). The same approach is possible between populations from Latvia and other Western European nations where unique environmental settings, distinct histories and relative isolation has led to the emergence of different genetic patterns within individual populations. The genetic patterns may have arisen so as to cope with the various socio-economic factors, pollution levels and lifestyle choices. The effect and contribution of each factor involved, whether genetic, lifestyle, or environmental, could therefore lead towards changes in CAD risk.

4.2 *GLO1* variants are strong determinants of glyoxalase activity

GLO1 is the frontline cellular defense against damage induced by AGEs. This protection is especially important in environments with elevated blood sugar levels such as that observed with diabetes and cells which use glucose as the main source of energy, such as neurons (Howarth *et al.* 2012). It has been shown that human neurons consume disproportionately large amounts of glucose relative to their mass (Mergenthaler *et al.* 2013). It is thus unsurprising that the main diabetes complications start with neuronal dysfunction such as neuropathic pain and diabetic retinopathy (Chilelli *et al.* 2013). The former condition affects the peripheral nerves in diabetic patients over time leading to pain, tingling, or numbness in the hands, arms, feet, and legs. The latter condition is the foremost cause of vision loss in diabetic individuals of all ages, and top cause of vision impairment and blindness among adults in the working-age group (The 2015). Yet, not all diabetes patients have these complications and those that do may manifest symptoms at different time points following the onset of diabetes. Disease progression rates may also vary between patients. A number of studies have been performed trying to link the genetics of *GLO1* with diabetes complications since *GLO1* is the main detoxification enzyme in the human body and diabetes complications are thought to be caused by AGEs. Given the amount of disorders where enzyme disruption is the leading cause of pathology, this is not surprising (National Organization for Rare Disorders 2006, The Genetic and Rare Diseases Information Center 2002). Results can however be inconsistent. For example the same, single study found that the rs2736654 A allele was not associated with nephropathy or retinopathy in an East Asian population, while rs1049346 was associated with both complications in patients with type 2

diabetes in the same population (Wu *et al.* 2011). Similarly, no associations were found between nine tag-SNPs in the *GLO1* gene and vascular complications in diabetes patients of European ancestry (Engelen *et al.* 2009). Results of our study did not show a direct association between the studied *GLO1* polymorphisms and diabetic neuropathy, nephropathy and retinopathy in either type 1 or type 2 diabetes patients. This leads to the obvious conclusion that the glyoxalase system is not the only factor influencing the development of diabetic complications, but add to the risks associated with this multifactorial disorder. Interestingly, the only SNP in our study that was not significantly associated with changes in enzymatic activity was found to change the qualitative composition of the enzyme. Although haplotype analysis data allows to hypothesize that it still modifies the effect of the both SNPs significantly associated to *GLO1* activity. Several studies have shown how intronic SNPs and SNPs present near genes can influence properties of the associated gene, and often properties of distant genes (Cooper 2010). Actually, instead of the more easily understandable changes in amino acid sequence of a protein/enzyme, SNPs found outside of gene coding regions are most commonly associated with a wide range of multifactorial disorders (Muller *et al.* 2016). Alternative splicing is a common mechanism by which noncoding SNPs could influence the amount or type of gene product made. In the case of *GLO1*, only one type of enzyme protein had been discovered in humans by 2017. Another transcript, which encompasses exons 4-6 and also includes rs1130534 (minor T allele associated with decreased GLO1 activity) and rs2736654 (missense SNP), constitutes a 7.27kb *GLO1* fragment. Another possibility is that one or both minor alleles of these SNPs disrupt the expression of *GLO1* leading to lower concentrations and activity of the enzyme.

Overall, it is likely that a lower activity of GLO1 in carriers of each of the minor alleles of the associated SNPs contributes towards the development of diabetic complications, but only in interaction with other environmental and genetic factors that modify the severity, timing and type of developing complication. Weaker enzyme action, together with other unbalanced elements, is likely to lead to more pronounced or earlier complications. To fully understand the involvement of *GLO1* and its interactions with the causes and complications of diabetes, considerable additional research and innovation is required to elucidate the molecular basis and modifying factors of diabetes.

Also it would be interesting to, in a prospective study, investigate if the alleles that contribute to the development of diabetic complications are significant determinants in the cause of death of T2D patients.

4.3 *SSTR5*, *MEN1* and *DRD2* contribute to pituitary adenoma development and associated clinical characteristics

Pituitary adenomas are benign tumors of the pituitary gland. The genetic cause of PA is heterogeneous and similar to that observed in most of the recognized multifactorial diseases. Classical subdivision of PA is based on the type of hormone/s secreted by the tumor. Functional PAs include prolactinomas, somatotropinomas, gonadotropinomas, TSH-secreting PAs, ACTH-secreting PAs, and PAs that secrete at least two hormones (mixed PAs). The remaining PAs are categorized as non-functional. This classification makes sense from a clinical point of view since hormonal imbalances lead to different clinical symptoms which require different treatment strategies. It becomes clear during the course of treatment that these broader categories encompass different types of PAs as the response to treatments and progression of neoplasms are often patient-specific. Also, when treating NFPAs, some patients occasionally respond to the treatment that is supposed to work on a functional PA type. This has led to the proposal that a molecular classification system of PA could help to improve the treatment and subsequently, the outcome of the disease. Under closer investigating of NFPA tissue samples through immunohistochemistry, some neoplasms showed that although hormones are produced, they are retained within cells and not secreted. In Latvia, patients are not always examined for the presence of gonadotropins due to limited funding for diagnostic tests. Therefore, these patients are usually classified alongside the NFPA group.

Monoclonality of PA was described during the 1990's; a fact that went unchecked with despite the availability of more modern genetic analysis methods (Jacoby *et al.* 1990).

While some genes causative of PA have been known for years (Pellegata *et al.* 2006, Stratakis and Ball 2000), genetic causes for different types of PA have only recently been discovered and reported (Beckers *et al.* 2015, Ma *et al.* 2015, Perez-Rivas *et al.* 2015, Reincke *et al.* 2015, Trivellin *et al.* 2014).

Studies performed by us have provided insight into several interesting genetic aspects of PA. The rationale behind the *SSTR5* studies was that *SSTR5* is expressed in somatotropinomas and, together with *SSTR2*, controls GH secretion and body growth (Panetta *et al.* 1994). At

the time the research was conducted, no studies had performed a comprehensive genetic investigation of *SSTR5* in cases of somatotropinomas.

In this first study, two variations in *SSTR5* (rs34037914 and rs642249) were associated with acromegaly-causing somatotropinomas.

The synonymous rs34037914 polymorphism was also associated with several independent characteristics that, when taken together, could clinically be described as more severe acromegaly. This was based on the finding that patients had a lower response to SA analogs, higher IGF-1 levels, and were more likely to have tumor surgery and recurrent PA surgery.

In the second study, six candidate genes (*SSTR2*, *DRD2*, *AIP*, *MEN1*, *GNAS*, *PRKAR1A*) were added in addition to the previously investigated *SSTR5* gene in studying the three most common types of PA (prolactinomas, NFPA and somatotropinomas).

When researching the genetic causes of PA, we favored an approach similar to that used in studies focused on other multifactorial diseases. This approach is justifiable since PA is common in the general population (Ezzat *et al.* 2004). However, only a small percentage of PA cases become clinically significant during the lifetime of the patients. This leads to the main problem associated with PA genetic studies which is the relative rarity of the clinically significant condition. Also, the availability of verified PA-free controls is a problem, as quick, cheap and patient-friendly tests do not exist. The best candidates for control samples would probably be patients with brain scans performed for other reasons (like stroke *etc.*).

The main discovery in the second study was the presence of rare, non-synonymous variation in the well-known PA gene – *MEN1*. The rs2959656 variant was found in seven PA cases and in only one control sample. While there was no predominant PA type associated with rs2959656, five of the carriers had clinically significant microadenomas. This suggests that PA is associated with this variant which either leads to an earlier presentation of PA symptoms or slow growing tumors. Although it is a missense variant, it is unlikely that rs2959656 alone is the cause of PA. It is a rare variant in populations of European descent, while it is fairly common in East Asian, American and African populations where it can reach a MAF of up to 0.40. We therefore propose that this variant is a marker for PA only in European populations, and further suggest that rs2959656 is either in LD with a causal variant, or works in synergy with additional variants in *MEN1* or genes in other locations. Alternatively, this variant may signal LOH in this region, which is a known cause of PA (Thakker 2014). LOH in this locus affects *MEN1* and *AIP* (Georgitsi *et al.* 2008).

Another interesting discovery was the association between two variants (rs7131056 and rs4938025) in the *DRD2* gene and the extrasellar growth of neoplasms in hormone-secreting PAs. Extrasellar growth is usually observed in macroadenomas and tumors that have faster expansion rates and more severe clinical symptoms (as classified under the Hardy PA classification system as grade III or IV). Extrasellar growth is the primary reason why the majority of NFPAs are discovered since the mass of the expanding tumor compresses the optical nerve, internal carotid arteries or other surrounding tissue. This may result in visual field or color vision disturbances, or more unspecific symptoms such as vertigo, nausea and headaches (Powell 2003). In our study, 77% of our NFPA samples consisted of tumors with extrasellar growth. In functional PAs, such as prolactinomas and somatotropinomas, the primary cause of pathology is the overproduction of hormones while the tumor itself is a rarer cause of complications. Regardless, 46% of functional PAs were placed in the extrasellar growth category. This could be due to a number of reasons. It could be because, by the time the patient undergoes pituitary imaging, there has been such an increase in the adenoma cell proliferation rate that the tumor has already attained the size used in the classification system. It could also be that a particular PA secretes lower amounts of hormones and therefore causes milder symptoms, subsequently delaying the diagnosis. Similarly, it could be that a cell in NFPA gives rise to a new secretory lineage and that symptoms of hormonal imbalance thus only start when the tumor is already large. Lastly, it is also possible that some of the patients delay seeking medical help after the onset of early symptoms. *DRD2* rs7131056, according to one report, was associated with social phobia (Sipila *et al.* 2010) giving some credence to the hypothesis of delayed visits to healthcare professionals. Other internal cellular mechanisms could also be involved. One example of this is when DRD2 physically forms heterodimers with SSTRs (Rocheville *et al.* 2000). As in most cases, the mechanism by which an intronic variant affects disease phenotype remains unclear (Cooper 2010). Seven variants of DRD2 protein have been found in humans and three more transcripts are made, two of them with retained intron where rs7131056 is located (Yates *et al.* 2016) providing range of possible mechanisms of action for the variant.

Direct comparison of both PA studies must be performed carefully due to the different scopes of the studies. The initial study was focused on identifying potential drivers of somatotropinoma in a subgroup of a well-characterized patient group. Various biochemical analyses and patient history details were available in this study. The second study in which seven candidate genes in three types of pituitary adenomas was the focus, common variants

predisposing patients to PA and its three predominant subtypes was searched for. A limited amount of additional phenotypic data was collected due to incomplete patient records. This design was taken in order to obtain larger sample sizes since this would increase the power of the study in detecting more modest associations, and increase the chances of capturing PA subgroups with a common driver or variant/mutation.

In the second study, the *SSTR5* rs34037914 variant remained statistically significant in the expanded study group (P=0,003 before correcting for multiple testing). Compared to the OR of 3.38 from the initial study (CI 0.95=1.78-6.42), the OR remained similar at 2.63 in the second study (CI 0.95=1.36-5.38). The association of rs34037914 with PA was not statistically significant after correcting for the 62 independent tests that were made during the study. However, the decrease in rs34037914 minor allele frequency in the expanded somatotropinoma patient group confirmed that this variant contributes towards clinically more severe acromegaly with a pronounced phenotype and more complications during the course of therapy. These patients were therefore recognized earlier and thus more likely to be included in the initial discovery study.

There were two more variants associated with the presence of acromegaly in the initial study. In the second study the rs642249 probe design was not working in the Illumina GoldenGate multiplex assay and no data was acquired. The *SSTR5* rs169068 variant was not statistically significant in the second study, thus providing further evidence that its association in the initial study was due to high LD with the two lead SNPs.

The initial somatotropinoma study raised awareness of PA and increased efforts were applied to contact endocrinologists outside of the Riga. This led to increased diagnosis rates of clinically milder forms of acromegaly and PA in general. This was probably also strengthened due to the relative depletion of obvious PA cases in the population as the study sample size increased.

4.4 Research strategies of molecular basis in multifactorial diseases

Understanding the molecular basis of multifactorial diseases and their complications will without a doubt lead to improved medical care. This may come in the form of novel drugs targeting newly discovered molecular pathways, an increase in treatments relating to gene expression, and possibly even gene editing therapies. In cases where the molecular basis of the disease is known, the main benefit for the patient would be precision medicine tailored to prevent the onset of the disease in the patient. Under such a model, the best available

therapy could be selected in order to manage disease prognosis and the likelihood of disease-associated complications.

The initial excitement about the use of genetic information to estimate the risk of developing multifactorial diseases receded after the first set of negative results. However, the most recent research showed that, if applied correctly together with conventional knowledge, the use of a patient's genetic information is possible and potentially beneficial. Together with the falling cost of genetic analysis it is also becoming a cost effective diagnostic option. The main advantage of genetic information is its relative stability and minimal changes throughout a person's lifetime, unlike blood biomarkers. It can also be used to assess multiple multifactorial diseases, drug responses and disease phenotypes thus increasing the cost-to-benefit ratio. Once new discoveries are made and verified, their information can be added to the known genetic data of a person without the need for the individual to visit a medical facility for check-ups or additional laboratory testing. Although it has been suggested that the strength of genotype–phenotype associations is modified by age (Genin *et al.* 2011), genetic markers are likely to be predictive throughout an individual's lifetime. This allows risk prediction to be performed much earlier in life, thus facilitating earlier primary prevention strategies in high-risk individuals.

Current research strategies investigating the molecular basis of multifactorial diseases consist of multiple complementary tiers. Traditionally, GWAS with large case and control groups are used to identify possible disease-associated markers. In order to validate findings, a second GWAS is preferably performed and, in an ideal scenario, it is performed in a different population thereby facilitating the reduction in the number of variants of interest. This is typically followed by a period where smaller studies from all over the world target candidate genes and try to replicate the association in separate populations. Compared to initial discovery studies, candidate gene studies usually use more phenotypic information to understand the finer details of the association in the samples. Candidate gene studies are interesting since significant precision medicine aspects can emerge. This is because authors frame the aims of the studies differently and stratify the samples using phenotypic combinations in order to uncover finer details about the associations. It is important to obtain sufficient statistical power in these follow-up studies in order to obtain conclusive results and preferably report on the negative associations too. Then, of course, there are functional studies that investigate mechanisms by which molecular variation is translated

into different processes within cells, organs and the whole organism, as well as how they interact with and respond to an environment.

Nowadays with the rapid advancement of DNA sequencing and editing technologies, the search for the molecular basis of multifactorial diseases has gained next level of momentum and the precision medicine is gaining an ever increasing importance in the modern biomedicine.

5. Conclusions

1. Minor allele of the adenosine A3 receptor polymorphism rs35511654 is protective against CAD in Latvian population under additive and dominant models.

2. Minor alleles of rs1130534 and rs1049346 in *GLO1* are associated with decreased Glo1 activity in Latvian population.

3. Minor allele of rs2736654 in *GLO1* is modulating observable GLO1 activity.

4. There is no association between type 1 or type 2 diabetes and their respective complications and the three GLO1 polymorphisms that were studied in Latvian population.

5. *SSTR5* polymorphisms rs34037914 and rs642249 are associated with the presence of acromegaly in Latvian population.

6. *MEN1* polymorphism rs2959656 is associated with the presence of clinically significant pituitary adenoma in Latvian population.

7. *DRD2* polymorphisms rs7131056 and rs4938025 are associated with the extrasellar growth of hormones secreting pituitary adenomas.

8. Germline polymorphisms of *SSTR2*, *AIP*, *GNAS* and *PRAKR1A* do not contribute to the development of PA in a significant proportion of patients in Latvian population.

6. Thesis for defense

1. Polymorphisms in the adenosine A3 receptor are minor contributors and modulators of disease and disease outcome, and are involved in the pathophysiology of CAD.

2. *GLO1* genetic variants are strong determinants of glyoxalase activity, but are not associated with diabetes complications in Latvian population.

3. *SSTR5* polymorphism rs34037914 determines the severity of GH-secreting adenoma cases diagnosed with acromegaly.

4. The *MEN1* polymorphism rs2959656 is a strong predictor of the clinically significant presence of pituitary adenoma in Latvian population.

5. Distinguishing between multifactorial disease sub-phenotypes improves the capacity to uncover new genetic factors.

List of Original Publications

1. Ciganoka, D., I. Balcere, I. Kapa, **R. Peculis**, A. Valtere, L. Nikitina-Zake, I. Lase, H. B. Schioth, V. Pirags and J. Klovins (2011). "Identification of somatostatin receptor type 5 gene polymorphisms associated with acromegaly." Eur J Endocrinol 165(4): 517-525.
2. **Peculis, R.**, I. Balcere, V. Rovite, K. Megnis, A. Valtere, J. Stukens, L. Arnicane, L. Nikitina-Zake, A. Lejnieks, V. Pirags and J. Klovins (2016). "Polymorphisms in MEN1 and DRD2 genes are associated with the occurrence and characteristics of pituitary adenomas." Eur J Endocrinol 175(2): 145-153.
3. **Peculis, R.**, I. Konrade, E. Skapare, D. Fridmanis, L. Nikitina-Zake, A. Lejnieks, V. Pirags, M. Dambrova and J. Klovins (2013). "Identification of glyoxalase 1 polymorphisms associated with enzyme activity." Gene 515(1): 140-143.
4. **Peculis, R.**, G. Latkovskis, L. Tarasova, V. Pirags, A. Erglis and J. Klovins (2011). "A nonsynonymous variant I248L of the adenosine A3 receptor is associated with coronary heart disease in a Latvian population." DNA Cell Biol 30(11): 907-911.
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10. Polymorphisms in FTO and near TMEM18 associate with type 2 diabetes and predispose to younger age at diagnosis of diabetes. Kalnina I, Zaharenko L, Vaivade I, Rovite V, Nikitina-Zake L, **Peculis R**, Fridmanis D, Geldnere K, Jacobsson JA, Almen MS, Pirags V, Schiöth HB, Klovins J. Gene. 2013 Sep 25;527(2):462-8. doi: 10.1016/j.gene.2013.06.079. Epub 2013 Jul 13.
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Approbation of the Research, Published Thesis

24.02.2017. 75th Scientific Conference of the University of Latvia International Medical Section, Significant association of four SNPs with lower age at diagnosis in patients with GH secreting pituitary adenoma, **Raitis Pečulis**, Inga Balcere, Andra Valtere, Ilze Konrāde, Olīvija Caune, Valdis Pīrāgs, Jānis Kloviņš

25.09.2016. – 28.09.2016. 14th International Workshop on Multiple Endocrine Neoplasia and other rare neuroendocrine tumors. **R. Peculis.**

12.04.2015. – 16.04.2015. 2nd Molecular Diagnostics Europe. Modeling fetal DNA fraction requirements for chr21, chr18 and chr13 trisomy detection using semiconductor sequencing. **Raitis Peculis**, Vita Rovite, Ilze Radovica, Davids Fridmanis, Ieva Grinfelde, Natalija Vedmedovska, Pavels Domasevs, Dace Rezeberga, Janis Klovins.

30.01.2015. 73rd LU conference, Neinvazīvas prenatālās testēšanas metodes izstrāde aneiploīdiju detektēšanai ar MPS, **Raitis Pečul**is, Vita Rovīte, Ilze Radoviča, Dāvids Fridmanis, Ieva Grīnfelde, Natālija Vedmedovska, Pāvels Domaševs, Dace Rezeberga, Jānis Kloviņš

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Decreased glyoxalase 1 activity caused by polymorphisms in *GLO1* is gender dependent,

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17.06.2012. – 22.06.2012. ICQG 2012. Decreased glyoxalase 1 activity caused by polymorphisms in GLO1 is gender dependent, **Raitis Peculis**, Ilze Konrade, Elina Skapare, Davids Fridmanis, Liene Nikitina-Zake, Aivars Lejnieks, Valdis Pirags, Maija Dambrova, Janis Klovins.

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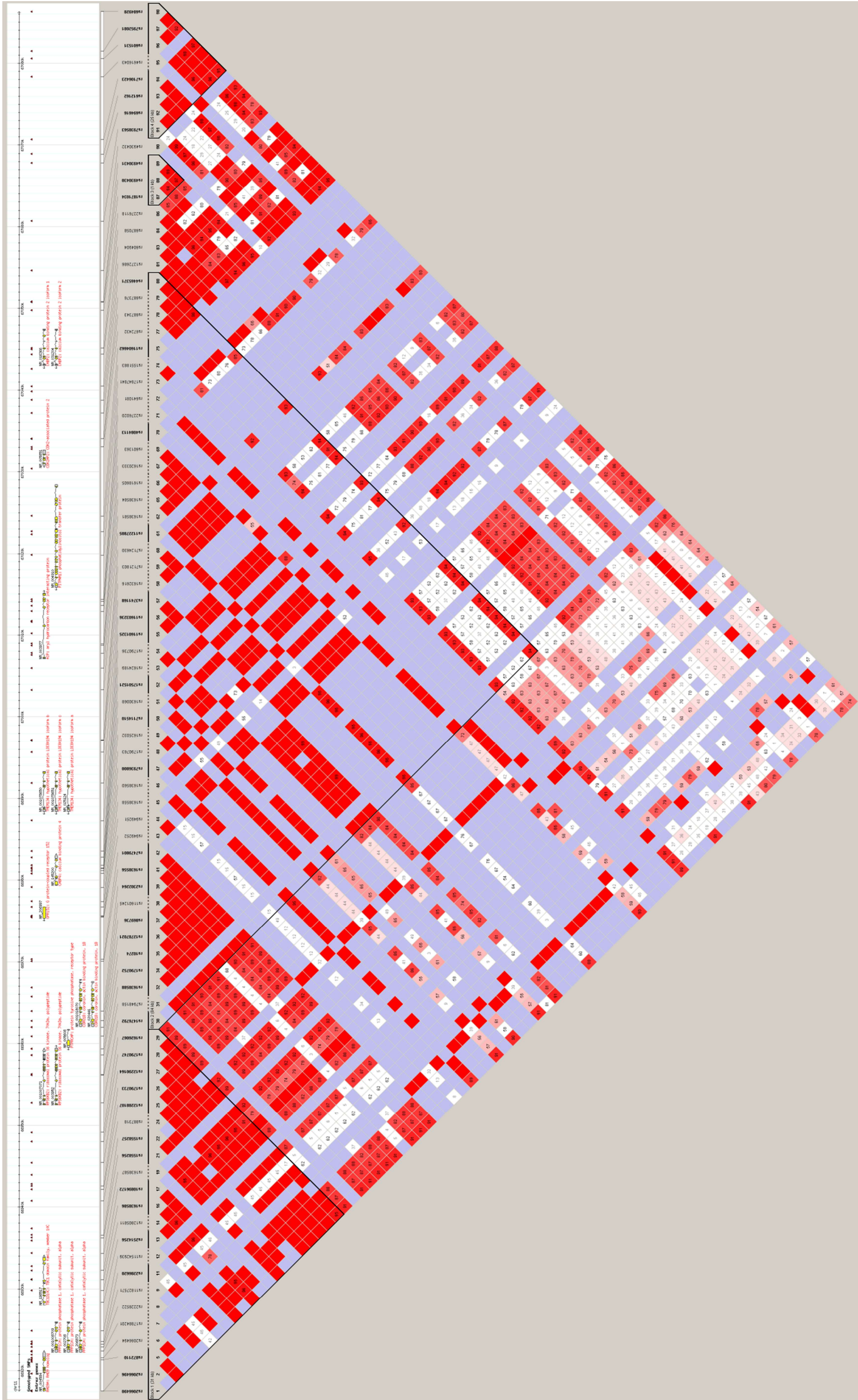
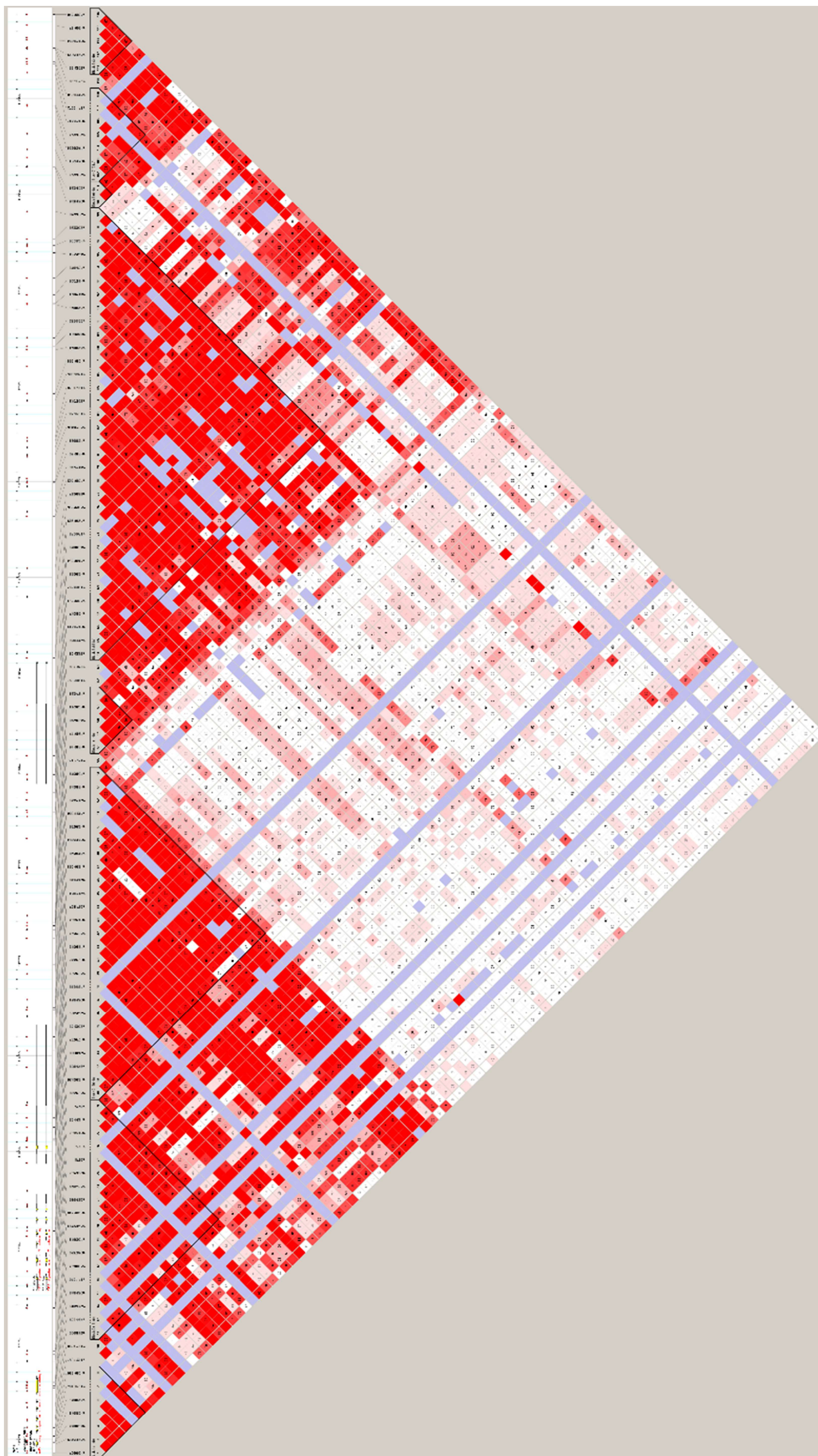
Supplementary

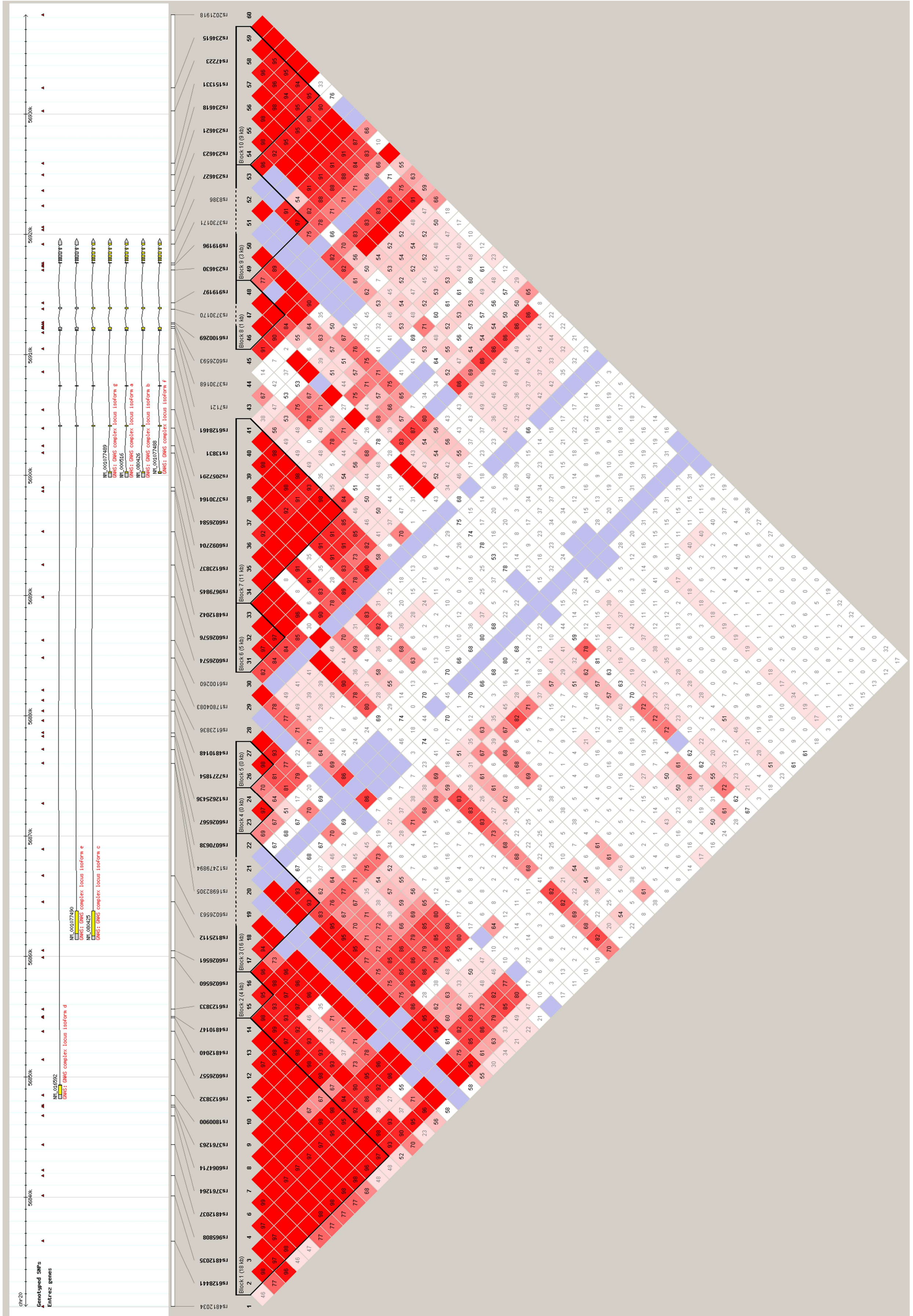
Supplementary 1.1. Linkage disequilibrium plot of *AIP* and +/- 70 kb region.



Supplementary 1.2. Linkage disequilibrium plot of *DRD2* and +20kb/- 70 kb region.

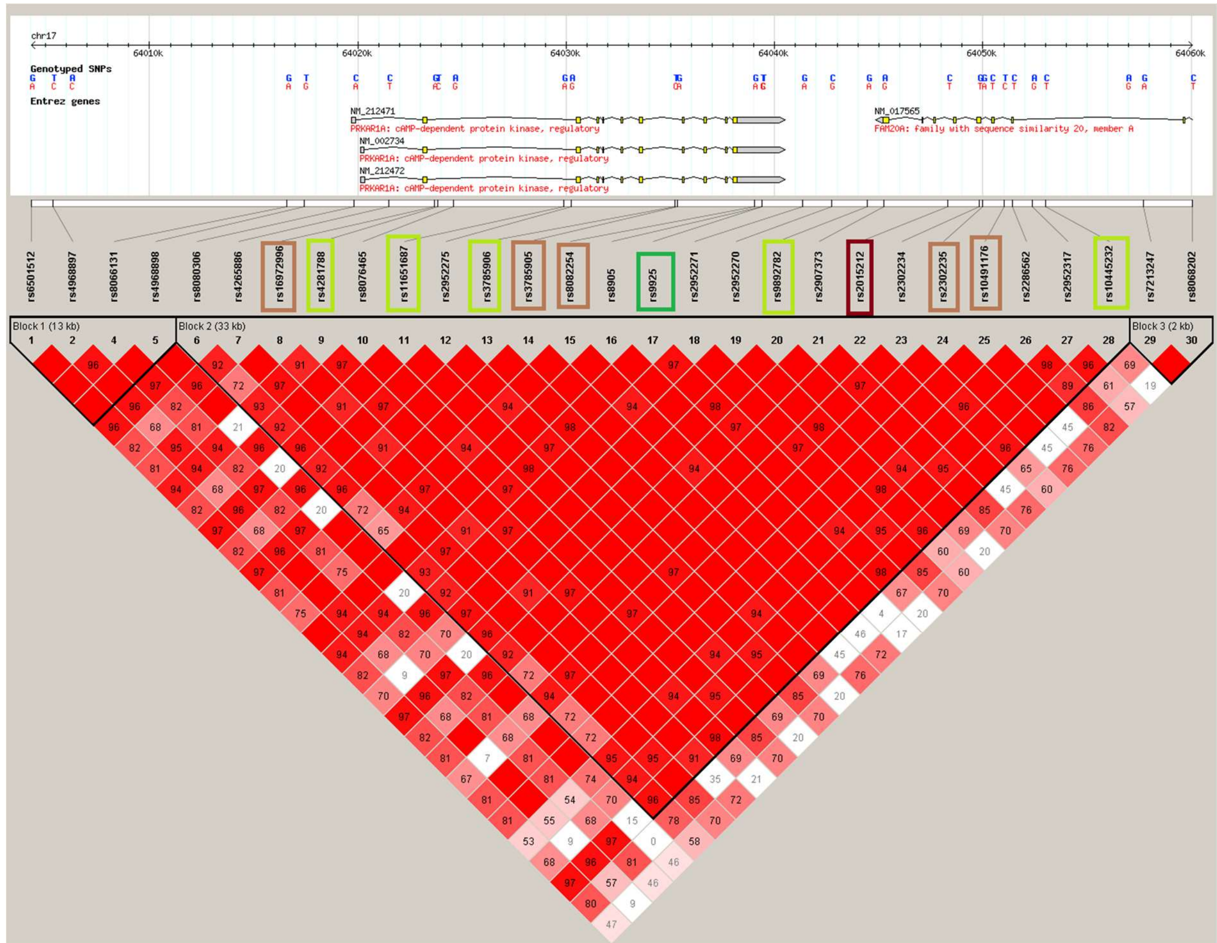


Supplementary 1.3. Linkage disequilibrium plot of *GNAS* and +/- 20 kb region.

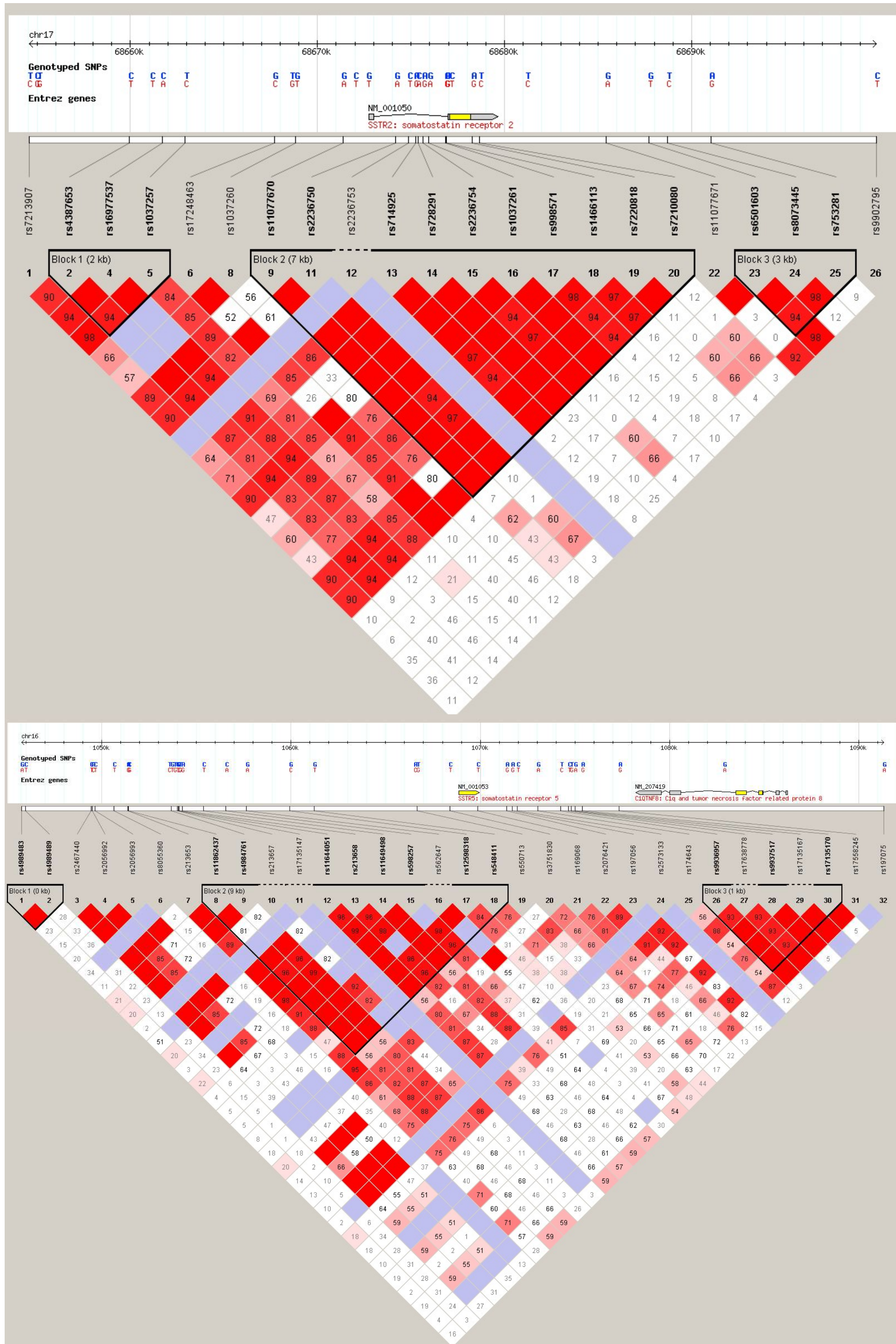


Supplementary 1.4. Linkage disequilibrium plot of *MEN1* and +/- 20 kb region.

Supplementary 1.5. Linkage disequilibrium plot of *PRKAR1A* and +/- 20 kb region.



Supplementary 1.6. Linkage disequilibrium plot of *SSTR2* (above) and *SSTR5* (below) and+/- 20 kb region.



Supplementary 2. Tag-SNPs used in pituitary adenoma study of 2016.

CHR	SNP	Gene	Gene context
11	rs1620333	AIP	1 intron
11	rs11214606	DRD2	1 intron
11	rs12805897	DRD2	1 intron
11	rs17529477	DRD2	1 intron
11	rs2471857	DRD2	1 intron
11	rs4936270	DRD2	1 intron
11	rs4938019	DRD2	1 intron
11	rs4938021	DRD2	1 intron
11	rs7125415	DRD2	1 intron
11	rs7131056	DRD2	1 intron
11	rs2002453	DRD2	2 intron
11	rs17847841	AIP	3' intergenic
11	rs687376	AIP	3' intergenic
11	rs10891549	DRD2	3' intergenic
11	rs12422191	DRD2	3' intergenic
11	rs2242592	DRD2	3' intergenic
11	rs2734848	DRD2	3' intergenic
11	rs2734849	DRD2	3' intergenic
11	rs7944809	DRD2	3' intergenic
11	rs11604662	AIP	3' nearby gene
11	rs2276020	AIP	syn D-D
11	rs2734838	DRD2	3 intron
11	rs624975	MEN1	3 intron
11	rs2440390	DRD2	4 intron
11	rs11603236	AIP	5' intergenic
11	rs1638558	AIP	5' intergenic
11	rs7114510	AIP	5' intergenic
11	rs7936800	AIP	5' intergenic
11	rs7948158	AIP	5' intergenic
11	rs10736466	DRD2	5' intergenic
11	rs10891564	DRD2	5' intergenic
11	rs12292637	DRD2	5' intergenic
11	rs17602285	DRD2	5' intergenic
11	rs4245154	DRD2	5' intergenic
11	rs4938025	DRD2	5' intergenic
11	rs4938026	DRD2	5' intergenic
11	rs6589382	DRD2	5' intergenic
11	rs11601245	AIP	5' nearby gene
11	rs1124492	DRD2	7 intron
11	rs504195	MEN1	7 intron
11	rs2959656	MEN1	missense A-T
11	rs61741147	AIP	missense A-V
11	rs1800497	DRD2	missense E-K

CHR	SNP	Gene	Gene context
11	rs607969	MEN1	missense R-Q
11	rs1801028	DRD2	missense S-C
11	rs6274	DRD2	3' UTR variant
11	rs2071313	MEN1	synonymous D-D
11	rs4986918	DRD2	synonymous L-L
16	rs550713	SSTR5	1 intron
16	rs197056	SSTR5	3' intergenic
16	rs2076421	SSTR5	3'mRNS
16	rs3751830	SSTR5	5' non coding transcript SSTR5
16	rs4988484	SSTR5	missense A-V
16	rs169068	SSTR5	missense P-L
16	rs4988487	SSTR5	missense P-S
16	rs34803442	SSTR5	missense V-M
16	rs34037914	SSTR5	synonymous F-F
16	rs642249	SSTR5	synonymous P-P
17	rs1466113	SSTR2	1 intron
17	rs714925	SSTR2	1 intron
17	rs2015212	PRKAR1A	3' intergenic
17	rs9925	PRKAR1A	3' mRNS
17	rs11077670	SSTR2	5' intergenic
17	rs2236753	SSTR2	SSTR2 processed transcript
17	rs61743817	SSTR2	missense S-F
17	rs7220818	SSTR2	3' UTR variant
20	rs6026557	GNAS	1 intron
20	rs6026560	GNAS	1 intron
20	rs6026561	GNAS	1 intron
20	rs16982305	GNAS	2 intron
20	rs17804083	GNAS	2 intron
20	rs4810148	GNAS	2 intron
20	rs6026563	GNAS	2 intron
20	rs6026567	GNAS	2 intron
20	rs6026574	GNAS	2 intron
20	rs6070638	GNAS	2 intron
20	rs6100260	GNAS	2 intron
20	rs7271854	GNAS	2 intron
20	rs8125112	GNAS	2 intron
20	rs234627	GNAS	3' intergenic
20	rs6123837	GNAS	3 intron
20	rs9679845	GNAS	3 intron
20	rs3730164	GNAS	4 intron
20	rs4812037	GNAS	5' intergenic
20	rs6064714	GNAS	5' intergenic
20	rs965808	GNAS	5' intergenic
20	rs2057291	GNAS	5 intron
20	rs13831	GNAS	6 intron

CHR	SNP	Gene	Gene context
20	rs3730168	GNAS	8 intron
20	rs3730170	GNAS	8 intron
20	rs6100269	GNAS	8 intron
20	rs919197	GNAS	8 intron
20	rs919196	GNAS	9 intron
20	rs11554273	GNAS	missense R-G
20	rs79709641	GNAS	missense R-H
20	rs7121	GNAS	synonymous I-I

Supplementary 3. Primers of SSTR5 amplification (A) and sequencing (B)

A. PCR

Oligonucleotide ID	Lenght (bp)	Sequence
IVK-03-Fw	22	GCATGTGCTGGTTCAGGGACTC
IVK-03-Rs	21	TGTGGCAGACGGTTAGAGGGG
STR5p1Fw	19	CCCTGCTCCAGTGAATCTT
STR5p1Rs	22	CTGAAATCTGGTAGACAAGGAA
STR5p2Fw	21	GGGGATGTTGATTTCTAGGTT
STR5p2Rs	20	GGAGGCTGAGTCAGGAGGAT
STR5p3Fw	20	GGCTGGCGTGCAGTGGTGTA
STR5p4Fw	18	AAGCTCCGATGGGACTGG
STR5p4Rs	18	CACCTGCGTCTCCCTGAA
STR5p5Fw	18	GCCTCGGCTCCTTACCTG
STR5p5Rs	19	CACCAGCGTCCTGTTGTCA

B. Sequencing

Oligonucleotide ID	Lenght (bp)	Sequence
IVK-03-Seq1f	20	CACCCTGGCGTCCTCCCTTC
IVK-03-Seq2f	22	ACGGCGTCAACCAGTTCACCAG
IVK-03-Seq3r	23	TGAGCAGGTAGCACAGGCAGATG
IVK-03-Seq4r	20	CACCGCAGTGCAACCTCCGC
STR5p1SFw	18	GTGAATCTTTCAACCCTC
STR5p1SRs	20	GTAGACAAGGAAAGGAGAGC
STR5p2SFw	18	ATTTCTAGGTTTGTTGAGC
STR5p2SRs	19	TCAGGAGGATCATTTGAGC
STR5p3SFw	18	CAGTGGTGTAATCACAGC
STR5p3SRs	18	AGAGGCATAGCGTATGGG
STR5p4SFw	18	GGGACTGGGCAGTGTCAG
STR5p4SRs	19	TCCCTGAAGCCCCTCAGAG
STR5p5SFw	18	CTTACCTGGTAGACGAGG
STR5p5SRs	19	CTGTTGTCACCGCCTCCAG

Supplementary 4. Mini-sequencing primers

Oligonucleotide ID	Lenght (bp)	Sekvence
JAK-28-MS	18	GGCCLTGCTGGGCTTCTT [C/T]
JAK-29.1-MS	19	CCGGATCLTGTCTGGACGC [A/G]
JAK-29.2-MS	19	AGCLGCAGGAGGCCACGCC [A/G]

Supplementary 5. Results of main GWAS of CAD published until 2017. CHR – chromosome, POS – position, SNP – single nucleotide polymorphism, RA – risk allele, RAF – risk allele frequency, OR – odds ratio, CI – confidence interval, CAD – coronary artery disease, MI – myocardial infarction, NR – not reported, UTR – untranslated region, nc – non-coding.

AUTHOR	Year	DISEASE	REGION	CHR_POS	MAPPED_GENE	SNP-RA	CONTEXT	RAF	OR	95% CI
Samani NJ	2007	CAD	6q25.1	150931849	MTHFD1L	rs6922269-A	intron	0,25	1,23	[1.15-1.33]
Samani NJ	2007	CAD	2q36.3	226203364	LOC646736	rs2943634-C	intergenic	0,65	1,21	[1.13-1.30]
WTCCC	2007	CAD	9p21.3	22125504	CDKN2B	rs1333049-C	downstream_gene	0,47	1,47	[1.27-1.70]
WTCCC	2007	CAD	1q43	240282296	FMN2	rs17672135-T	intron	0,87	1,43	[1.23-1.64]
WTCCC	2007	CAD	22q12.1	26293669	SEZ6L	rs688034-T	intron	0,31	1,11	[0.99-1.25]
WTCCC	2007	CAD	16q23.3	83178793	CDH13	rs8055236-G	intron	0,80	1,91	[1.33-2.74]
Samani NJ	2007	CAD	1q41	222650187	MIA3	rs17465637-C	intron	0,71	1,2	[1.12-1.30]
Samani NJ	2007	CAD	9p21.3	22125504	CDKN2B	rs1333049-C	downstream_gene	0,47	1,36	[1.27-1.46]
Samani NJ	2007	CAD	1p13.3	109279544	CELSR2	rs599839-A	downstream_gene	0,77	1,29	[1.18-1.40]
Samani NJ	2007	CAD	10q11.21	44258419	LINC00841	rs501120-T	downstream_gene	0,87	1,33	[1.20-1.48]
Samani NJ	2007	CAD	15q22.33	67166301	SMAD3	rs17228212-C	intron	0,30	1,21	[1.13-1.30]
Helgadottir	2007	MI	9p21.3	22124478	CDKN2B	rs10757278-G	downstream_gene	0,45	1,28	[1.22-1.35]
Erdmann J	2009	CAD	3q22.3	138403280	MRAS	rs9818870-T	3_prime_UTR	0,15	1,15	[1.11-1.19]
Erdmann J	2009	CAD	12q24.31	120997784	HNF1A	rs2259816-T	synonymous	0,36	1,08	[1.05-1.11]
Kathiresan S	2009	MI	9p21.3	22098575	CDKN2B	rs4977574-G	intron	0,56	1,29	[1.25-1.34]
Kathiresan S	2009	MI	1p13.3	109275908	CELSR2	rs646776-T	downstream_gene	0,81	1,19	[1.13-1.26]
Kathiresan S	2009	MI	1q41	222650187	MIA3	rs17465637-C	intron	0,72	1,14	[1.10-1.19]
Kathiresan S	2009	MI	10q11.21	44280376	LINC00841	rs1746048-C	downstream_gene	0,84	1,17	[1.11-1.24]
Kathiresan S	2009	MI	21q22.11	34226827	LINC00310	rs9982601-T	intron	0,13	1,2	[1.14-1.27]
Kathiresan S	2009	MI	6p24.1	12927312	PHACTR1	rs12526453-C	intron	0,65	1,12	[1.08-1.17]
Kathiresan S	2009	MI	2q33.2	202881162	WDR12	rs6725887-C	intron	0,14	1,17	[1.11-1.23]
Kathiresan S	2009	MI	19p13.2	11052925	SMARCA4	rs1122608-G	intron	0,75	1,15	[1.10-1.20]
Kathiresan S	2009	MI	1p32.3	55030366	BSND	rs11206510-T	intergenic	0,81	1,15	[1.10-1.21]
Tregouet D	2009	CAD	6q25.3	160541471	LPA	rs7767084-T	intron	0,16	1,2	[1.13-1.28]

AUTHOR	Year	DISEASE	REGION	CHR_POS	MAPPED_GENE	SNP-RA	CONTEXT	RAF	OR	95% CI
Tregouet	2009	CAD	6q25.3	160442500	SLC22A3	rs2048327-C	intron	0,02	1,82	[1.57-2.12]
Erdmann J	2010	CAD	10p11.23	30027143	KIAA1462	rs3739998-C	missense	0,44	1,15	[1.11-1.20]
Aoki A	2010	MI	5p15.33	4029676	LOC105374626	rs11748327-?	downstream_gene	NR	1,25	[1.18-1.33]
Schunkert H	2011	CAD	1p32.3	55030366	BSND	rs11206510-T	intergenic	0,82	1,08	[1.05-1.11]
Schunkert H	2011	CAD	10q11.21	44280376	LINC00841	rs1746048-C	downstream_gene	0,87	1,09	[1.07-1.13]
Schunkert H	2011	CAD	1p32.2	56497149	PPAP2B	rs17114036-A	intron	0,91	1,17	[1.13-1.22]
Schunkert H	2011	CAD	6p21.31	35067023	ANKS1A	rs17609940-G	intron	0,75	1,07	[1.05-1.10]
Schunkert H	2011	CAD	6q23.2	133893387	TCF21	rs12190287-C	3_prime_UTR	0,62	1,08	[1.06-1.10]
Schunkert H	2011	CAD	7q32.2	130023656	ZC3HC1	rs11556924-C	missense	0,62	1,09	[1.07-1.12]
Schunkert H	2011	CAD	9q34.2	133278724	ABO	rs579459-C	upstream_gene	0,21	1,1	[1.07-1.13]
Schunkert H	2011	CAD	10q24.32	102959339	CNNM2	rs12413409-G	intron	0,89	1,12	[1.08-1.16]
Schunkert H	2011	CAD	11q23.3	116778201	ZPR1	rs964184-G	3_prime_UTR	0,13	1,13	[1.10-1.16]
Schunkert H	2011	CAD	13q34	110308365	COL4A2	rs4773144-G	intron	0,44	1,07	[1.05-1.09]
Schunkert H	2011	CAD	14q32.2	99667605	HHIPL1	rs2895811-C	intron	0,43	1,07	[1.05-1.10]
Schunkert H	2011	CAD	15q25.1	78796769	ADAMTS7	rs3825807-A	missense	0,57	1,08	[1.06-1.10]
Schunkert H	2011	CAD	17p13.3	2223210	SMG6	rs216172-C	intron	0,37	1,07	[1.05-1.09]
Schunkert H	2011	CAD	17p11.2	17640408	PEMT	rs12936587-G	intergenic	0,56	1,07	[1.05-1.09]
Schunkert H	2011	CAD	17q21.32	48911235	UBE2Z	rs46522-T	nc_transcript_exon	0,53	1,06	[1.04-1.08]
C4D	2011	CAD	9p21.3	22098575	CDKN2B	rs4977574-G	intron	NR	1,2	[1.16-1.25]
C4D	2011	CAD	1p13.3	109275908	CELSR2	rs646776-T	downstream_gene	NR	1,14	[1.09-1.19]
C4D	2011	CAD	6p24.1	12888772	PHACTR1	rs1332844-T	intron	NR	1,11	[1.07-1.15]
C4D	2011	CAD	10q23.31	89243170	LIPA	rs1412444-T	intron	0,42	1,09	[1.07-1.12]
C4D	2011	CAD	11q22.3	103789839	LOC105369463	rs974819-T	intron	0,32	1,07	[1.04-1.09]
C4D	2011	CAD	15q25.1	78818751	ADAMTS7	rs4380028-C	intron	0,65	1,07	[1.05-1.10]
C4D	2011	CAD	7q22.3	107604100	BCAP29	rs10953541-C	intron	0,80	1,08	[1.05-1.11]
C4D	2011	CAD	10p11.23	30046193	KIAA1462	rs2505083-C	intron	0,38	1,07	[1.04-1.09]
C4D	2011	CAD	6p24.1	12903725	PHACTR1	rs9349379-?	intron	NR		
C4D	2011	CAD	Xq23	110695977	CHRDL1	rs5943057-?	intron	NR		

AUTHOR	Year	DISEASE	REGION	CHR_POS	MAPPED_GENE	SNP-RA	CONTEXT	RAF	OR	95% CI
C4D	2011	CAD	4q31.22	147126398	LOC105377476	rs1395821-?	intergenic	NR		
C4D	2011	CAD	15q24.1	74741059	CYP1A1	rs2472299-?	intergenic	NR		
C4D	2011	CAD	2q32.1	187331742	LOC105373786	rs840616-?	intron	NR		
C4D	2011	CAD	17q12	33960430	LOC107985038	rs11650066-?	intron	NR		
C4D	2011	CAD	17q23.3	64330939	PECAM1	rs6504218-?	intron	NR		
C4D	2011	CAD	2q32.1	187478770	LOC105373786	rs7586970-?	missense	NR		
C4D	2011	CAD	1p32.2	56500678	PPAP2B	rs17114046-?	intron	NR		
C4D	2011	CAD	9q22.33	97977842	HEMGN	rs4743150-?	intergenic	NR		
Schunkert H	2011	CAD	1p13.3	109279544	CELSR2	rs599839-A	downstream_gene	0,78	1,11	[1.08-1.15]
Schunkert H	2011	CAD	1q41	222650187	MIA3	rs17465637-C	intron	0,74	1,14	[1.09-1.20]
Schunkert H	2011	CAD	2q33.2	202881162	WDR12	rs6725887-C	intron	0,15	1,14	[1.09-1.19]
Schunkert H	2011	CAD	3q22.3	138401110	MRAS	rs2306374-C	intron	0,18	1,12	[1.07-1.16]
Schunkert H	2011	CAD	6p24.1	12927312	PHACTR1	rs12526453-C	intron	0,67	1,1	[1.06-1.13]
Schunkert H	2011	CAD	6q25.3	160540105	LPA	rs3798220-C	missense	0,02	1,51	[1.33-1.70]
Schunkert H	2011	CAD	9p21.3	22098575	CDKN2B	rs4977574-G	intron	0,46	1,29	[1.23-1.36]
Schunkert H	2011	CAD	12q24.12	111446804	SH2B3	rs3184504-T	missense	0,44	1,07	[1.04-1.10]
Schunkert H	2011	CAD	19p13.2	11052925	SMARCA4	rs1122608-G	intron	0,77	1,14	[1.09-1.18]
Schunkert H	2011	CAD	21q22.11	34226827	LINC00310	rs9982601-T	intron	0,15	1,18	[1.12-1.24]
Schunkert H	2011	CAD	2q37.1	233133771	INPP5D	rs10933436-A	intron	0,49	1,06	[1.04-1.09]
Schunkert H	2011	CAD	3p25.1	15606497	BTD	rs7651039-C	nc_transcript_exon	0,54	1,06	[1.04-1.09]
Schunkert H	2011	CAD	7q31.2	117427768	ASZ1	rs7808424-G	intron	0,12	1,1	[1.06-1.14]
Schunkert H	2011	CAD	11q24.2	126412002	ST3GAL4	rs4937126-G	intron	0,69	1,06	[1.04-1.09]
Schunkert H	2011	CAD	17p13.3	2222311	SMG6	rs1231206-A	intron	0,37	1,07	[1.05-1.09]
C4D	2011	CAD	10q24.32	102959339	CNNM2	rs12413409-?	intron	NR		
Wild PS	2011	CAD	9p21.3	22125504	CDKN2B	rs1333049-C	downstream_gene	0,49	1,27	[1.23-1.31]
Wild PS	2011	CAD	9p21.3	22031006	CDKN2B	rs7865618-A	intron	0,59	1,18	[1.14-1.21]
Wild PS	2011	CAD	10q23.31	89243170	LIPA	rs1412444-T	intron	0,32	1,1	[1.07-1.14]
Wild PS	2011	CAD	6q25.3	159225301	FNDC1	rs365302-C	intron	0,24	1,11	[1.06-1.15]

AUTHOR	Year	DISEASE	REGION	CHR_POS	MAPPED_GENE	SNP-RA	CONTEXT	RAF	OR	95% CI
Wild PS	2011	CAD	6q14.1	81805598	LOC105377873	rs16893526-G	intergenic	0,91	1,13	[1.07-1.21]
Slavin TP	2011	CAD	1q21.1	146039391	TRH	rs12091564-C	upstream_gene	NR		
Slavin TP	2011	CAD	3q26.1	162443828	LOC107986049	rs11924705-C	intergenic	NR		
Slavin TP	2011	CAD	4p16.2	5366258	STK32B	rs7697839-G	intron	NR		
Slavin TP	2011	CAD	9p21.3	22125504	CDKN2B	rs1333049-G	downstream_gene	NR		
Slavin TP	2011	CAD	12q23.3	103924394	GNN	rs1165668-G	intron	NR		
Slavin TP	2011	CAD	1q21.1	146018957	HFE2	rs10218795-C	intron	NR		
Slavin TP	2011	CAD	3q26.1	162449608	LOC107986049	rs6789378-A	intergenic	NR		
Slavin TP	2011	CAD	4p16.2	5366497	STK32B	rs7673097-G	intron	NR		
Slavin TP	2011	CAD	9p21.3	22125348	CDKN2B	rs1333048-G	downstream_gene	NR		
Slavin TP	2011	CAD	12q23.3	103924218	GNN	rs1165668-G	intron	NR		
Mehta NN	2011	CAD	6p24.1	12903725	PHACTR1	rs9349379-?	intron	NR		
Mehta NN	2011	CAD	1p32.2	56500678	PPAP2B	rs17114046-?	intron	NR		
Takeuchi F	2011	CAD	6p21.32	32701596	HLA	rs11752643-T	downstream_gene	0,06	1,26	[1.15-1.38]
Takeuchi F	2011	CAD	9p21.3	22115287	CDKN2B	rs944797-C	intron	0,46	1,25	[1.18-1.31]
Takeuchi F	2011	CAD	12q24.12	111803962	ALDH2	rs671-A	missense	0,23	1,43	[1.35-1.51]
Davies RW	2012	CAD	6p21.33	31216419	HCG27	rs3869109-G	regulatory_region	NR	1,14	[NR]
Davies RW	2012	CAD	1q21.3	154465420	IL6R	rs2229238-?	3_prime_UTR	NR	1,45	[NR]
Davies RW	2012	CAD	6p21.1	43791136	VEGFA	rs6905288-T	downstream_gene	NR	1,23	[NR]
Davies RW	2012	CAD	6q16.1	96632322	FHL5	rs12200560-?	intergenic	NR	1,11	[NR]
Davies RW	2012	CAD	2q35	215420652	FN1	rs17458018-?	intron	NR	1,22	[NR]
Hager J	2012	CAD	6p24.1	12903725	PHACTR1	rs9349379-G	intron	0,35	1338	[1.22-1.47]
Hager J	2012	CAD	6p24.1	12903725	PHACTR1	rs9349379-G	intron	0,35	1187	[1.22-1.44]
Hager J	2012	CAD	6p24.1	12903725	PHACTR1	rs9349379-G	intron	0,35	1,23	[1.14-1.33]
Lu X	2012	CAD	2p24.1	19745816	LOC105373461	rs2123536-T	intergenic	0,39	1,12	[1.08-1.16]
Lu X	2012	CAD	4q32.1	155590307	LOC105377504	rs1842896-T	intergenic	0,76	1,14	[1.10-1.19]
Lu X	2012	CAD	6p21.32	32373576	LOC101929163	rs9268402-G	upstream_gene	0,59	1,16	[1.12-1.20]
Lu X	2012	CAD	12q21.33	89687411	ATP2B1	rs7136259-T	intron	0,39	1,11	[1.08-1.15]

AUTHOR	Year	DISEASE	REGION	CHR_POS	MAPPED_GENE	SNP-RA	CONTEXT	RAF	OR	95% CI
Lu X	2012	CAD	9p21.3	22096056	CDKN2B	rs10757274-G	intron	0,46	1,37	[1.31-1.43]
Lu X	2012	CAD	12q24.13	112379979	HECTD4	rs11066280-A	intron	0,17	1,19	[1.13-1.25]
Lu X	2012	CAD	6p24.1	12903725	PHACTR1	rs9349379-G	intron	0,74	1,15	[1.10-1.21]
Lu X	2012	CAD	6q23.2	133875536	TARID	rs12524865-C	intron	0,61	1,11	[1.06-1.16]
Lee JY	2013	CAD	12q24.11	111241410	CUX2	rs886126-T	intron	0,66	1,14	[1.08-1.20]
Lee JY	2013	CAD	12q24.11	110912851	MYL2	rs3782889-C	intron	0,21	1,26	[1.19-1.34]
Lee JY	2013	CAD	13q12.3	28409926	FLT1	rs9508025-C	intron	0,50	1,14	[1.08-1.20]
Lee JY	2013	CAD	9p21.3	22103814	CDKN2B	rs1333042-?	intron	NR	1,3	[1.19-1.41]
Lee JY	2013	CAD	12q24.12	111730205	ACAD10	rs11066015-A	intron	NR	1,41	[1.27-1.56]
Hirokawa M	2014	MI	3p24.3	17082892	PLCL2	rs4618210-G	intron	0,44	1,1	[1.06-1.14]
Hirokawa M	2014	MI	19p13.3	2160530	AP3D1	rs3803915-C	intron	0,19	1,12	[1.09-1.16]
Hirokawa M	2014	MI	9p21.3	22098575	CDKN2B	rs4977574-C	intron	0,46	1,22	[1.12-1.33]
Hirokawa M	2014	MI	12q24.12	111672685	BRAP	rs3782886-A	synonymous	0,26	1,46	[1.33-1.60]
Reilly MP	2011	CAD	15q25.1	78787892	ADAMTS7	rs1994016-C	intron	0,60	1,19	[1.13-1.24]
Reilly MP	2011	MI in CAD				rs514659-C		0,37	1,21	[1.13-1.29]
Reilly MP	2011	CAD	1p13.3	109275908	CELSR2	rs646776-T	downstream_gene	0,79	1,33	[1.22-1.44]
Reilly MP	2011	CAD	9p21.3	22098575	CDKN2B	rs4977574-G	intron	0,53	1,3	[1.22-1.39]
Lettre G	2011	CAD	5q31.3	142484039	LOC101926941	rs17577085-?	intron	0,46	2,63	[1.72-3.85]
Lettre G	2011	CAD	7q36.1	151423862	WDR86	rs13232179-A	upstream_gene	0,11	1,67	[1.36-2.06]
Lettre G	2011	CAD	13q31.1	84480131	LINC00333	rs9546711-A	intergenic	0,42	1,66	[1.35-2.04]
Nikpay M	2015	CAD	4q12	56972417	NOA1	rs17087335-T	intron	0,21	1,06	[1.04-1.09]
Nikpay M	2015	CAD	7q36.1	150993088	NOS3	rs3918226-T	intron	0,06	1,14	[1.09-1.19]
Nikpay M	2015	CAD	15q22.33	67163292	SMAD3	rs56062135-C	intron	0,79	1,07	[1.05-1.10]
Nikpay M	2015	CAD	15q26.1	89030987	LOC100129942	rs8042271-G	intergenic	0,90	1,1	[1.06-1.14]
Nikpay M	2015	CAD	17q23.2	60936127	BCAS3	rs7212798-C	intron	0,15	1,08	[1.05-1.11]
Nikpay M	2015	CAD	18q21.32	60171168	RNU4	rs663129-A	intergenic	0,26	1,06	[1.04-1.08]
Nikpay M	2015	CAD	22q11.23	24262890	BCRP1	rs180803-G	intron	0,97	1,2	[1.13-1.27]

AUTHOR	Year	DISEASE	REGION	CHR_POS	MAPPED_GENE	SNP-RA	CONTEXT	RAF	OR	95% CI
Nikpay M	2015	CAD	19q13.11	32391114	LOC400684	rs12976411-A	intron	0,09	1,49	[1.35-1.67]
Nikpay M	2015	CAD	12q24.23	117827636	KSR2	rs11830157-G	intron	0,36	1,12	[1.08-1.16]
Nikpay M	2015	CAD	11p15.4	9729649	SWAP70	rs10840293-A	intron	0,55	1,06	[1.04-1.08]
Nikpay M	2015	CAD	9p21.3	22098620	CDKN2B	rs2891168-G	intron	0,49	1,21	[1.19- 1.24]
Nikpay M	2015	CAD	6p24.1	12903725	PHACTR1	rs9349379-G	intron	0,43	1,14	[1.12- 1.16]
Nikpay M	2015	CAD	6q25.3	160584578	LPA	rs55730499-T	intron	0,06	1,37	[1.31- 1.44]
Nikpay M	2015	CAD	6q26	160702419	PLG	rs4252185-C	intron	0,06	1,34	[1.28- 1.41]
Nikpay M	2015	CAD	1p13.3	109274570	CELSR2	rs7528419-A	3_prime_UTR	0,79	1,12	[1.10- 1.15]
Nikpay M	2015	CAD	15q25.1	78832133	LOC390614	rs4468572-C	intron	0,59	1,08	[1.06- 1.10]
Nikpay M	2015	CAD	21q22.11	34221526	LINC00310	rs28451064-A	intron	0,12	1,14	[1.10- 1.17]
Nikpay M	2015	CAD	19p13.2	11077571	SMARCA4	rs56289821-G	intergenic	0,90	1,14	[1.11- 1.18]
Nikpay M	2015	CAD	10q11.21	43985363	LINC00841	rs1870634-G	intergenic	0,64	1,08	[1.06- 1.10]
Nikpay M	2015	CAD	1p32.2	56499992	PPAP2B	rs9970807-C	intron	0,92	1,13	[1.10- 1.17]
Nikpay M	2015	CAD	1q41	222650401	MIA3	rs67180937-G	intron	0,66	1,08	[1.06- 1.11]
Nikpay M	2015	CAD	10q23.31	89243170	LIPA	rs1412444-T	intron	0,37	1,07	[1.05- 1.09]
Nikpay M	2015	CAD				rs2519093-T		0,19	1,08	[1.06- 1.11]
Nikpay M	2015	CAD	6q23.2	133852013	LINC01312	rs12202017-A	intron	0,70	1,07	[1.05- 1.09]
Nikpay M	2015	CAD	10p11.23	30034963	KIAA1462	rs2487928-A	intron	0,42	1,06	[1.04- 1.08]
Nikpay M	2015	CAD	7q32.2	130023656	ZC3HC1	rs11556924-C	missense	0,69	1,08	[1.05- 1.10]
Nikpay M	2015	CAD	12q21.33	89615182	ATP2B1	rs2681472-G	intron	0,20	1,08	[1.05- 1.10]
Nikpay M	2015	CAD	11q22.3	103802549	LOC105369463	rs2128739-A	intron	0,32	1,07	[1.05- 1.09]
Nikpay M	2015	CAD	19q13.32	44919689	APOC1	rs4420638-G	downstream_gene	0,17	1,1	[1.07- 1.13]
Nikpay M	2015	CAD	7p21.1	19009765	HDAC9	rs2107595-A	regulatory_region	0,20	1,08	[1.05- 1.10]
Nikpay M	2015	CAD	13q34	110388334	COL4A2	rs11838776-A	intron	0,26	1,07	[1.05- 1.09]
Nikpay M	2015	CAD	2p11.2	85561052	GGCX	rs7568458-A	intron	0,45	1,06	[1.04- 1.08]
Nikpay M	2015	CAD	4q31.22	147359849	MIR548G	rs4593108-C	intergenic	0,80	1,07	[1.05- 1.10]
Nikpay M	2015	CAD	12q24.12	111446804	SH2B3	rs3184504-T	missense	0,42	1,07	[1.04- 1.09]
Nikpay M	2015	CAD	1q21.3	154423470	IL6R	rs6689306-A	intron	0,45	1,06	[1.04- 1.08]

AUTHOR	Year	DISEASE	REGION	CHR_POS	MAPPED_GENE	SNP-RA	CONTEXT	RAF	OR	95% CI
Nikpay M	2015	CAD	2q22.3	144528992	LINC01412	rs17678683-G	upstream_gene	0,09	1,1	[1.07- 1.14]
Nikpay M	2015	CAD	10q24.32	102845159	PFN1P11	rs11191416-T	intron	0,87	1,08	[1.05- 1.11]
Nikpay M	2015	CAD	4q32.1	155718736	GUCY1A3	rs72689147-G	intron	0,82	1,07	[1.05- 1.10]
Nikpay M	2015	CAD	14q32.2	99679373	HHIPL1	rs10139550-G	3_prime_UTR	0,42	1,06	[1.04- 1.08]
Nikpay M	2015	CAD	2p24.1	19742712	LOC105373461	rs16986953-A	intergenic	0,10	1,09	[1.06- 1.12]
Nikpay M	2015	CAD	6p21.2	39166323	SAYSD1	rs56336142-T	regulatory_region	0,81	1,07	[1.04- 1.09]
Nikpay M	2015	CAD	1p32.3	55030366	BSND	rs11206510-T	intergenic	0,85	1,08	[1.05- 1.11]
Nikpay M	2015	CAD	15q26.1	90873320	FURIN	rs17514846-A	intron	0,44	1,05	[1.03- 1.07]
Nikpay M	2015	CAD	17p13.3	2223210	SMG6	rs216172-C	intron	0,35	1,05	[1.03- 1.07]
Nikpay M	2015	CAD	8q24.13	125478730	TRIB1	rs2954029-A	intron	0,55	1,04	[1.03- 1.06]
Nikpay M	2015	MI	10q24.32	102834750	CYP17A1	rs1004467-A	nc_transcript_exon	0,87	1,08	[1.05-1.11]
Nikpay M	2015	MI	14q32.2	99679373	HHIPL1	rs10139550-G	3_prime_UTR	0,41	1,05	[1.03-1.07]
Nikpay M	2015	MI	2p11.2	85534925	RPSAP22	rs10176176-T	upstream_gene	0,47	1,07	[1.05-1.09]
Nikpay M	2015	MI	6q25.3	160589086	LPA	rs10455872-G	intron	0,05	1,33	[1.27-1.4]
Nikpay M	2015	MI	1p32.3	55030366	BSND	rs11206510-T	intergenic	0,85	1,08	[1.05-1.11]
Nikpay M	2015	MI	7q32.2	130023656	ZC3HC1	rs11556924-C	missense	0,70	1,07	[1.05-1.1]
Nikpay M	2015	MI	13q34	110165755	COL4A1	rs11617955-T	intron	0,90	1,09	[1.05-1.12]
Nikpay M	2015	MI	1q21.3	154424940	IL6R	rs12118721-T	intron	0,48	1,06	[1.04-1.08]
Nikpay M	2015	MI	10q23.31	89243662	LIPA	rs1332329-C	intron	0,36	1,08	[1.06-1.1]
Nikpay M	2015	MI	6p21.2	39156672	SAYSD1	rs1544935-T	intergenic	0,81	1,08	[1.05-1.10]
Nikpay M	2015	MI	2p24.1	19742712	LOC105373461	rs16986953-A	intergenic	0,09	1,08	[1.05-1.12]
Nikpay M	2015	MI	2q22.3	144528992	LINC01412	rs17678683-G	upstream_gene	0,09	1,08	[1.05-1.11]
Nikpay M	2015	MI	22q11.23	24262890	BCRP1	rs180803-G	intron	0,97	1,21	[1.14-1.28]
Nikpay M	2015	MI	10q11.21	43985363	LINC00841	rs1870634-G	intergenic	0,62	1,07	[1.05-1.09]
Nikpay M	2015	MI	8q24.13	125466208	TRIB1	rs2001846-T	upstream_gene	0,49	1,05	[1.03-1.07]
Nikpay M	2015	MI	11q22.3	103798234	LOC105369463	rs2019090-A	intron	0,36	1,07	[1.05-1.09]
Nikpay M	2015	MI	6q26	160687112	LPA	rs2315065-A	intergenic	0,06	1,3	[1.24-1.36]
Nikpay M	2015	MI	10p11.23	30046193	KIAA1462	rs2505083-C	intron	0,39	1,06	[1.04-1.08]

AUTHOR	Year	DISEASE	REGION	CHR_POS	MAPPED_GENE	SNP-RA	CONTEXT	RAF	OR	95% CI
Nikpay M	2015	MI	15q26.1	90894158	FES	rs2521501-T	intron	0,29	1,07	[1.04-1.09]
Nikpay M	2015	MI	12q21.33	89615182	ATP2B1	rs2681472-G	intron	0,19	1,08	[1.05-1.1]
Nikpay M	2015	MI	21q22.11	34221526	LINC00310	rs28451064-A	intron	0,12	1,13	[1.09-1.17]
Nikpay M	2015	MI	9p21.3	22098620	CDKN2B	rs2891168-G	intron	0,48	1,21	[1.19-1.23]
Nikpay M	2015	MI	1q41	222638065	MIA3	rs35700460-G	intron	0,65	1,09	[1.06-1.11]
Nikpay M	2015	MI	17q21.32	48982960	GIP	rs35895680-C	intergenic	0,72	1,06	[1.03-1.08]
Nikpay M	2015	MI	4q31.22	147359849	MIR548G	rs4593108-C	intergenic	0,80	1,07	[1.04-1.09]
Nikpay M	2015	MI				rs532436-A		0,19	1,12	[1.09-1.14]
Nikpay M	2015	MI	19p13.2	11077477	SMARCA4	rs55791371-A	intergenic	0,90	1,11	[1.07-1.15]
Nikpay M	2015	MI	13q34	110390962	COL4A2	rs55940034-G	intron	0,27	1,07	[1.04-1.09]
Nikpay M	2015	MI	19q13.32	44919589	APOC1	rs56131196-A	downstream_gene	0,16	1,09	[1.06-1.12]
Nikpay M	2015	MI	12q24.12	111569952	ATXN2	rs653178-C	intron	0,44	1,08	[1.06-1.1]
Nikpay M	2015	MI	15q25.1	78830996	ADAMTS7	rs7165042-C	intron	0,56	1,06	[1.04-1.09]
Nikpay M	2015	MI	17q23.2	60936127	BCAS3	rs7212798-C	intron	0,15	1,07	[1.04-1.1]
Nikpay M	2015	MI	4q32.1	155718736	GUCY1A3	rs72689147-G	intron	0,82	1,08	[1.05-1.1]
Nikpay M	2015	MI	15q22.33	67149412	SMAD3	rs72743461-C	intron	0,80	1,07	[1.05-1.1]
Nikpay M	2015	MI	1p13.3	109274570	CELSR2	rs7528419-A	3_prime_UTR	0,80	1,11	[1.08-1.13]
Nikpay M	2015	MI	6p24.1	12903725	PHACTR1	rs9349379-G	intron	0,41	1,14	[1.12-1.16]
Nikpay M	2015	MI	17p13.3	2229956	SMG6	rs9914266-C	intron	0,35	1,05	[1.03-1.07]
Nikpay M	2015	MI	1p32.2	56499992	PPAP2B	rs9970807-C	intron	0,92	1,12	[1.08-1.16]
Dehghan A	2016	CAD	13q31.3	91102945	LINC00380	rs16945184-C	intergenic	NR	1,20	[1.11-1.3]
Dehghan A	2016	CAD	6p21.2	40414972	LRFN2	rs2916260-T	intron	NR	1,15	[1.09 - 1.22]
Dehghan A	2016	MI	6q26	163609768	QKI	rs6941513-G	intergenic	NR	1,2	[1.13-1.28]
Dehghan A	2016	MI	4q35.1	182703491	TENM3	rs7692395-G	intron	NR	1,45	[1.27-1.69]
Dehghan A	2016	MI	7p21.2	14815437	DGKB	rs4721377-T	intron	NR	1,26	[1.14-1.38]
Dehghan A	2016	MI	16q24.1	86651813	FOXL1	rs4843416-G	intron	NR	1,43	[1.22-1.67]
Wakil SM	2016	CAD	2q33.1	198223806	PLCL1	rs7421388-G	intron	0,79	1,30	[NR]
Wakil SM	2016	CAD	3q25.31	156287165	KCNAB1	rs13082914-T	intron	0,22	1,21	[NR]

AUTHOR	Year	DISEASE	REGION	CHR_POS	MAPPED_GENE	SNP-RA	CONTEXT	RAF	OR	95% CI
Wakil SM	2016	CAD	4q31.23	150108918	DCLK2	rs9985766-G	intron	0,16	1,35	[NR]
Wakil SM	2016	CAD	5q11.2	52889863	ITGA1	rs16880442-G	nc_transcript_exon	0,93	1,39	[NR]
Wakil SM	2016	CAD	6q25.3	156266980	LOC101928923	rs17775862-T	intergenic	0,06	1,55	[NR]
Wakil SM	2016	CAD	8q21.11	72230147	LOC392232	rs12541758-T	nc_transcript_exon	0,40	1,25	[NR]
Wakil SM	2016	CAD	9p21.3	22088095	CDKN2B	rs10738607-G	intron	0,64	1,28	[NR]
Wakil SM	2016	CAD	9q31.3	111690726	C9orf84	rs10981012-A	intron	0,13	1,34	[NR]
Wakil SM	2016	CAD	10q11.21	44280862	LINC00841	rs1746049-C	downstream_gene	0,72	1,25	[NR]
Wakil SM	2016	MI	3q25.1	149875124	RNF13	rs41411047-A	intron	0,09	1,51	[NR]
Wakil SM	2016	MI	4q31.23	150108918	DCLK2	rs9985766-G	intron	0,16	1,23	[NR]
Wakil SM	2016	MI	5p13.3	32073460	PDZD2	rs32793-G	intron	0,28	1,25	[NR]
Wakil SM	2016	MI	5q11.2	52889863	ITGA1	rs16880442-G	nc_transcript_exon	0,93	1,41	[NR]
Wakil SM	2016	MI	8q12.3	63191534	YTHDF3	rs4739066-A	intron	0,86	1,37	[NR]
Wakil SM	2016	MI	8q21.11	72230147	LOC392232	rs12541758-T	nc_transcript_exon	0,40	1,16	[NR]
Wakil SM	2016	MI	9p21.3	22088095	CDKN2B-AS1	rs10738607-G	intron	0,63	1,27	[NR]
Wakil SM	2016	MI	17q25.3	80133738	GAA	rs7211079-A	downstream_gene	0,77	1,30	[NR]
Wakil SM	2016	MI	21q22.11	34226827	LINC00310	rs9982601-T	intron	0,16	1,38	[NR]