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Functionality and genetics of melanocortin and purinergic receptors

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ABSTRACT

Melanocortin (MCR) and purinergic (P2YR) receptors are two distinct subgroups of G protein-coupled receptors (GPCRs). Natural ligands of MCRs are peptides while P2YRs are activated by small nucleotide molecules. The members of these receptor families are considered to be potential therapeutic targets. The possible involvement of MCRs and P2YRs in development of multifactorial diseases has been disputed. The aim of this study was to explore functional characteristics important for receptor activation of the two GPCR subgroups with structurally different natural ligand types using a yeast expression system and to investigate the implication of MCRs and P2YRs in multifactorial conditions using a genetic association approach. The construction and functional screening of a randomised library of melanocortin 4 receptor (MC4R) at the position 126, showed that aspartic acid in this position is crucial for receptor signalling and for constitutive activity of MC4R. The common SNP rs17782313 of *MC4R* in case-control groups of a Latvian population showed no association with obesity. However, functional testing of four nonsynonymous substitutions V103I, S127L, V166I and I251L found in morbidly obese group revealed that the novel substitution V166I cause decreased receptor quantity at the cell surface, but induced a hyperactive satiety signal transduction. S127L alone or in combination with V103I led to a dramatic decrease in receptor quantity in plasma membrane and inactivation. V103I and I251L did not affect MC4R signalling when stimulated by agonist, but AGRP demonstrated stronger inhibition of V103I variant activation compared to wild type MC4R. The designed study groups were also used for genotyping of the common fat mass and obesity-associated protein gene (*FTO*). Polymorphisms rs11642015 and rs62048402 were associated with obesity. The polymorphisms in the purinergic 1 receptor gene (*P2RY1*) locus were not associated with myocardial infarction or related phenotypes (body mass index, type 2 diabetes, *angina pectoris*, hypertension, hyperlipidemia, atrial fibrillation and heart failure) in case-control groups of a Latvian population. Detailed analysis of the regions involved in functional activity of purinergic 12 receptor (P2Y12R) demonstrated E181, R256, R265 and K280 to be important for signalling integrity of the receptor. Furthermore, tree-dimensional (3D) modelling studies suggested K280 to be a key determinant of the P2Y12R ADP binding pocket.

KOPSAVILKUMS

Melanokortīnu (MCR) un purīnu (P2YR) receptori ir divas atšķirīgas ar G proteīnu saistīto receptoru apakšgrupas. MCR dabīgie ligandi ir peptīdi, bet P2YR aktivējas piesaistot mazas nukleotīdu molekulas. Abu šo receptoru grupu pārstāvji tiek uzskatīti par potenciāliem terapeitiskiem mērķiem, kā arī plaši tiek apspriesta MCR un P2YR loma dažādu multifaktoriālu slimību attīstībā. Šī darba mērķis bija pētīt funkcionāli raksturīgās pazīmes, kas ir būtiskas receptoru aktivācijā divās GPCR apakšgrupās ar strukturāli atšķirīgiem dabīgo ligandu veidiem, lietojot raugu ekspresijas sistēmu, un pētīt MCR un P2YR nozīmību multifaktoriālajās slimībās ar ģenētiskās asociācijas metodēm. Darba gaitā izveidota un funkcionāli analizēta melanokortīnu 4. receptora (MC4R) D126. pozīcijas randomizētā bibliotēka, kas parādīja šīs pozīcijas nozīmību receptora signāla pārneses un konstitutīvās aktivitātes nodrošināšanā. Netika atrasta asociācija starp gēna *MC4R* polimorfismu rs17782313 un aptaukošanos Latvijas populācijas *case-control* grupās. Tomēr četru nesinonīmu nomaiņu - V103I, S127L, V166I un I251L, kas atrastas adipozo pacientu grupā, funkcionālā raksturošana parādīja, ka pirmo reizi detektētā izmaiņa V166I izraisa hiperaktīvu sāta signāla transdukciju, S127L nomaiņa (gan atsevišķi, gan kombinācijā ar V103I) dramatiski samazina receptora daudzumu plazmatiskajā membrānā un signāla transdukcijas aktivitāti. Izmaiņu V103I un I251L stimulācija ar agonistu neietekmēja MC4R funkcionalitāti, tomēr AGRP inhibīcija bija spēcīgāka V103I variantam nekā savvaļas tipa MC4R. Šīs pašas paraugkopas tika izmatotas arī ar adipozo audu masas un adipozitāti saistītā proteīna (FTO) gēna bieži sastopamo polimorfismu genotipēšanai, rs11642015 un rs62048402 uzrādīja asociāciju ar aptaukošanos. Ģenētiskā saistība netika atrasta starp purīnu 1. receptora gēna (*P2RY1)* lokusu un miokarda infarktu, kā arī ar citiem saistītiem fenotipiem (ķermeņa masas indeksu, otrā tipa diabētu, stenokardiju, hipertensiju, hiperlipidēmiju, priekškambaru mirdzēšanu un sirds mazspēju). Detalizēta receptora purīnu 12. receptora (P2Y12R) rajonu analīze norāda, ka E181, R256, R265 un K280 pozīcijām ir nozīmīga loma receptora funkciju nodrošināšanā, kā arī 3D modelēšana norāda uz K280 pozīcijas izšķirošo lomu ADP piesaistē pie receptora.

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ABBREVIATIONS

A#R	adenosine receptor (abbreviation according to IUPHAR database)
aa#	amino acid position (for example, K20 – lysine in position 20)
aa#aa	replacement of amino acid in position by other amino acid (for example, K20L – lysine in position 20 replaced by leucine)
ABCB#	ATP-binding cassette, sub-family B gene
ACTH	adrenocorticotropic hormone
ADP	adenosine diphosphate
AGRP	agouti-related protein
AKAP	A-kinase anchor proteins
AP-2	activating protein 2
AT	3-amino-1,2,4-triazole
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
BHK	baby hamster kindey
BMI	body mass index
CAD	coronary artery disease
CCR#	chemokine receptor
cGMP-PDE	cyclic guanosine monophosphate phosphodiesterase
Cdc#	cell division control protein
CNS	central nervous system
CTX	cholera toxin
CYP#	cytochrome P450 enzyme gene
DNA	deoxyribonucleic acid
DREADD	designer receptors exclusively activated by a designer drug
EC_{50}	half maximal effective concentration
EL#	extracellular loop
ER	endoplasmic reticulum
ERK	extracellular-signal-regulated kinase
FRET	fluorescence resonance energy transfer
FTO	fat mass and obesity-associated protein (if given in italic (*FTO*) then FTO gene)
GABAb	γ-aminobutyric acid b receptor
GAP	guanosine triphosphatases activating protein
GEF	guanine nucleotide exchange factor
GDP	guanosine diphosphate
GPCR	G protein coupled receptor
GRIN1	glutamate receptor subunit zeta-1
GRK	G protein coupled receptor kinase
GTP	guanosine triphosphate
GWAS	genome-wide association studies
H2	haplotype 2
HEK	human embryonic kidney
HSP#	heat shock protein
ICD	International Classification of Diseases
IL#	intracellular loop
iNOS	inducible nitric oxide synthases
LGDB	Latvian Genome Data Base
LD	linkage disequilibrium
MAF	minor allele frequency
MAP	mitogen-activated protein kinases
MC#R	melanocortin receptor
MC#R	melanocortin receptor gene (abbreviation according to IUPHAR database)
2-MeSADP	2-methylthio- adenosine diphosphate
MI	myocardial infarction
MRAP	melanocortin receptor accessory protein
MRS#	N-methanocarba-2-methylthio-ADP
MSH	melanocyte stimulating hormone
NDP-MSH	Nle4, D-Phe7-α- melanocyte stimulating hormone
NHE	sodium/hydrogen exchanger
OR	odds ratio
P2RY#	gene of purinergic receptor (abbreviation according to IUPHAR database)
P2XR	purinergic receptor (ion channel)
P2Y# R	purinergic receptor (G protein coupled)
pCMBS	p-chloromercuribenzene sulfonate
PDB	protein data bank
PI-3	phosphoinositol 3 phosphate
PLCβ	phospholipase Cβ
PKA	protein kinase A
POMC	proopiomelanocortin
PTX	pertussis toxin
P-Rex	guanine nucleotide exchange factor
QSAR	quantitative structure–activity relationship
Rac	subfamily of the Rho family of GTPases
RASSL	receptors activated solely by synthetic ligands
Rap	Ras (small GTPases)-related protein
Ras	small GTPases
Rho	Ras (small GTPases) homolog
rs#	reference single-nucleotide polymorphism code
SNP	single-nucleotide polymorphism
Src	proto-oncogene tyrosine-protein kinase
STREGA	strengthening the reporting of genetic associations
T2D	type 2 diabetes
THIQ	tetrahydroisoquinolinium derivate
TM#	transmembrane domain
UDP	uridine diphosphate
UTP	uridine triphosphate
VASP	vasodilator-stimulated phosphoprotein
wt	wild type
YM254890	a cyclic depsipeptide isolated from Chromobacterium sp QS3666
2D	two dimensional
3D	three dimensional

INTRODUCTION

The G protein-coupled receptors (GPCRs) have been implicated in various disease-related processes. The molecular biology, genetics, pharmacology and physiology of the GPCRs have been investigated for diagnostics, prognostics and therapeutic strategies for disease treatments. However, because of the complex functionality of the receptor signalling systems, many important features that could influence further development of medical innovations are not fully understood.

Melanocortin 4 receptor (MC4R) is a significant regulator of feeding behaviour. Genetic variation in the *MC4R* locus has been associated with various obesity-related conditions and amino acid substitutions in the coding region of the receptor cause severe monogenic obesity, even in heterozygous cases. Although, the genetics of *MC4R* have been extensively explored, research studies of different populations and cohorts have revealed more interesting and surprising insights about *MC4R* involvement in adiposity development. Functional studies of MC4R have described signalling specificity and the number of amino acids responsible for ligand-binding pocket formation. Novel MC4R ligands that could be used for adiposity treatment have been developed, but so far no receptor agonists have been investigated in clinical trials. Therefore, further research on MC4R functional regions could aid in targeted design of new anti-obesity drugs.

The P2Y12R is a crucial regulator of platelet aggregation and several anticoagulants (clopidogrel, ticlopidine) act as inhibitors on the P2Y12R. P2Y12R antagonists are used after different coronary interventions to protect against thrombus formation, but resistance to clopidogrel is seen in approximately one-third of patients. Attempts to develop other P2Y12R ligands are in progress. Developing novel ligands requires understanding the mechanisms of receptor activation and signal transduction. The regions of P2Y12R involved in receptor functions are poorly described in the literature, and new knowledge on this topic is needed.

Another P2Y12R related regulator of blood coagulation is P2Y1R. This GPCR is not a target of currently used medicines, but genetic variation in the gene *P2RY1* has been implicated in several diseases and the pharmacodynamics of aspirin and clopidogrel. However, no study has associated *P2RY1* polymorphisms with thrombus-related diseases.

Saccharomyces cerevisiae and mammalian cell lines are the two most commonly used expression systems in GPCR studies. Although yeast are considered a less sensitive model system than mammalian cells, unlike mammalian cells *Saccharomyces cerevisiae* is more isolated and contains no endogenous receptors and other factors that can increase background signalling. However, so far yeast has been used more often for expression of GPCRs that are activated by small ligands. The ability of large polypeptide ligands to penetrate through the polysaccharide rich cell wall of the *Saccharomyces cerevisiae* has not been assessed.

The aim of this thesis was to explore the functional characteristics that are important for receptor activation of the two GPCR subgroups with structurally different natural ligand types by exploiting the advantages of a yeast expression system, and investigate the implication of melanocortin and purinergic receptors (MCRs, P2YRs respectively) in multifactorial disorders using a genetic association approach. To accomplish the aim of the study following tasks were set:

1. Optimise MC4R expression in yeast *Saccharomyces cerevisiae* system, generate the randomized library at the D126 position of the MC4R and explore the influence of different amino acids at this position on receptor activation;
2. Optimise P2Y12R expression in *Saccharomyces cerevisiae* system, generate the randomised libraries of the E181, R256, R265 and K280 positions of the P2Y12R and investigate the role of these positions in receptor activation by bioinformatics analysis and three-dimensional modelling;
3. Evaluate and compare the expression and pharmacological characteristics of MCR and P2YR in the yeast system with the different types of natural ligands;
4. Study the possible role of *P2RY1* polymorphisms in development and pathogenesis of myocardial infarction and related conditions in the population of Latvia using a case-control approach;
5. Study the *MC4R* genetics to compare the impact of rare mutations and common polymorphisms in pathogenesis of extreme obesity in the population of Latvia;
6. Functionally characterise discovered nonsynonymous variants in the *MC4R*;
7. Study the common *FTO* polymorphisms in designed cohorts of morbidly obese and normal weight individuals in the population of Latvia.

1 LITERATURE REVIEW

1.1 G protein-coupled receptors

The G protein-coupled receptors (GPCR) constitute the largest cell surface receptor family. Estimates are that 1% of mammalian DNA encodes GPCRs and 800 GPCR sequences have been discovered in the human genome (Fredriksson *et al.*, 2003; Fredriksson *et al.*, 2005). Additionally, over than 30% of current drugs used in clinical practice take their action via GPCRs (Hill, 2006).

GPCRs are coupled to G proteins - guanine nucleotide binding proteins and their main function in signalling depends on guanosine diphosphate (GDP) substitution with guanosine triphosphate (GTP). This substitution further triggers second messenger pathways in the cell, regulating many functions that are vitally important for the organism (Alberts *et al.*, 2002).

1.1.1 Structure and classification of GPCRs

GPCRs bind various ligands with different chemical properties, for example, peptides, proteins, lipids, amines, nucleotides, ions, and even photons. Despite this great variation in signalling molecules, the structure of all GPCRs is remarkably similar (Fredriksson *et al.*, 2005).

The first evidence on the structure of GPCRs came in the beginning of the 1980s with the sequencing of bovine rhodopsin. Researchers found seven hydrophobic domains proposed to be located in the cell membrane (Hargrave *et al.*, 1983). GPCRs are composed of seven transmembrane (TM) α-helices with intracellular and extracellular loops (IL and EL) between them. The N-terminus of these receptors is located in the extracellular space, while the C-terminus is on the intracellular side of the cell membrane (Alberts *et al.*, 2002). After 10 years, the two-dimensional (2D) structure was resolved by electron crystallography and published (Schertler *et al.*, 1993), indicating the position and orientation of the TM domains. Finally, in 2000, the first 3D high-resolution model of bovine rhodopsine X-ray structure was reported (Palczewski *et al.*, 2000). This model served as the only template for homology modelling of other GPCRs until 2007, when the crystal structure of human β2 adrenergic receptor was published (Rasmussen *et al.*, 2007). In 2008, the structure of another GPCR - the adenosine 2a receptor (A2aR) - became

available (Jaakola *et al.*, 2008). Comparison between these models revealed that rhodopsin forms a rigid extracellular structure composed of the N-terminus and EL, while the extracellular site of β2 adrenergic and A2aR is more open and flexible. Currently, these three structures are used for homology modelling of other GPCRs and template choice highly depends on homology and ligand similarity characteristics between available templates and the target receptor (Tate *et al.*, 2009).

However, specific features of the GPCR structures can be learned not only from modelling, but also from alignment of receptor sequences. For example, since the rhodopsin-like receptors constitute almost 90% of all GPCRs, Mirzadegan and colleagues attempted to find commonalities in the rhodopsin receptor family structure using a multiple sequence alignment of 270 receptors. This study has revealed some interesting insights. First, the authors discovered highly conservative amino acid residues in the receptor helices: Gly and Asn in TM1, Leu and Asp in TM2, Cys and DRY motif in TM3, Trp and Pro in TM4, Pro and Tyr in TM5, Phe, Trp and Pro in TM6 and an NPXXXY motif in TM7. Although DRY and NPXXXY motifs have been described before, observations on other residues are highly valuable for further understanding GPCR signalling and can serve in homology modelling of GPCR structures. For example, the residue following Cys in the TM3 predicted the ligand type of the receptor. If Cys is followed by a basic amino acid such as Lys or Arg, the natural ligands of the receptor are most likely peptides. However, if Cys is followed by acidic residues such as Asp or Glu, the receptor has a pharmacological profile for biogenic amines (Mirzadegan *et al.*, 2003).

Attempts to classify GPCRs started in the early 1990s. At that time, sufficient information had accumulated to allow the recognition of similarities in the structures and functions of GPCRs. However, initially, researchers encountered the problem that GPCRs sharing common functions had very low amino acid sequence similarity, leading to rejection of the phylogenetic classification approach. The first classification system was described in 1994 (Attwood *et al.*, 1994). According to this system, all GPCRs discovered at that time were divided into six classes: A (rhodopsin), B (secretin), C (metabotropic glutamates/pheromone), D (fungal pheromone), E (cyclic adenosine monophosphate (cAMP) receptors), F (frizzled/smoothened). Authors defined these classes as clans, emphasising the common evolutionary ancestry of these receptors despite their diverse functional characteristics. Currently, the term

"family" is used more often. Later this classification system was defined as PRINTS, due to the use of specific patterns or "fingerprints" for receptor group recognition. Fingerprints are set of motifs that distinguish sequence region that are characteristic for a superfamily, family, or individual receptor, allowing the classification to be more thorough (Attwood *et al.*, 2002a; Attwood *et al.*, 2002b).

Despite the doubts in sequence alignment-based approaches for GPCR classification, in 2003, and alternative system called GRAFS was published, based on phylogenetic sequence analysis. GRAFS grouped GPCRs into five distinct families - glutamates, rhodopsins, adhesions, frizzled/taste 2 and secretin. The drawback of GRAFS was that it concentrated on human GPCRs, although correlation with the previous ABCDEF classification was also observed (Fredriksson *et al.*, 2003; Fredriksson *et al.*, 2005). The GRAFS rhodopsin class corresponded to group A, glutamate to C and fizzled to F from the ABCDEF classification; however, adhesions and secretin receptors were designated in separate groups while in the conventional system they both constitute class B (Davies *et al.*, 2007). Comparison between the classification systems is in Table 1.

Table 1. Classification of GPCRs by ABCDEF and by GRAFS systems (adapted from Davies et al. 2007)

ABCDEF classification	ABCDEF family description	GRAFS classification	GRAFS family description
A	Rhodopsin-like	R	Rhodopsin
B	Secretin-like	A	Adhesion
		S	Secretin
C	Metabotropic glutamates/pheromone	G	Glutamate
D	Metabotropic glutamates/pheromone	-	-
E	cAMP receptors	-	-
F	Frizzled/Smoothened	F	Frizzled
-	-	F	Taste2

Classification systems have greatly aided the annotation of orphan GPCR receptors that have been discovered solely by sequence alignment techniques. Knowledge about the functions of orphan receptors is usually limited, therefore, classification into distinct GPCR groups has promoted the guided exploration of their function and "deorphanization" (Davies *et al.*, 2007; Fredriksson *et al.*, 2003).

1.1.2 Signal transduction mechanism and trafficking

Signal transduction via GPCRs begins with the ligand binding to the receptor. Depending on the biophysical properties of the receptor, the ligand can either bind deeply between the TM helices, or some peptide ligands have been shown to bind also extracellular loops. Large hormones can bind to the N-terminus, which then directs the appropriate region of the hormone to interact with the receptor binding site (Oldham *et al.*, 2008).

Ligand binding leads to a conformational change in the TM helices of the receptor that result in heterotrimeric G-protein binding to the receptor in the intracellular space (Alberts *et al.*, 2002). Heterotrimeric G proteins consist of three G protein subunits, α, β and γ. In the human genome, G-proteins 35 genes have been discovered: 16 encode 21 Gα subunits, 5 encode 6 β subunits and 14 code an γ subunit, but only 12 G-protein subunits have been described in detail (Milligan *et al.*, 2006). Characterization of G-protein subunits is in Table 2.

Table 2. Characteristics of G protein subunits (adapted from Milligan et al. 2006)

Type	Subtype	Effectors	Expression	Bacterial toxins
$G\alpha_s$	$G\alpha_{s(S)}$ $G\alpha_{s(L)}$ $G\alpha_{s(XL)}$ $G\alpha_{olf}$	Adenylyl cyclases ↑ ($G\alpha_{s,s(XL),olfa}$) Maxi K channel ↑ ($G\alpha_s$) Src tyrosine kinases (c-Src, Hck) ↑ ($G\alpha_s$) GTPase of tubulin ↑ ($G\alpha_s$)	$G\alpha_s$: ubiquitous $G\alpha_{olf}$: olfactory neurons, certain CNS ganglia; digestive and urogenital tract	$G\alpha_s$: CTX $G\alpha_{olf}$: CTX
$G\alpha_{i/0}$	$G\alpha_{o1}$ $G\alpha_{o2}$ $G\alpha_{i1–i3}$ $G\alpha_z$ $G\alpha_{t1/2}$ $G\alpha_{gust}$	Adenylyl cyclase ↓ ($G\alpha_{i,o,z}$) Rap1GAPII-dependent ERK/MAPkinase activation ↑ ($G\alpha_i$) Ca^{2+} channels ↓ ($G\alpha_{i,o,z}$) K^+ channels ↑ ($G\alpha_{i,o,z}$) GTPase of tubulin ↑ ($G\alpha_i$) Src tyrosine kinases (c-Src, Hck) ↑ ($G\alpha_i$) Rap1GAP ↑ ($G\alpha_z$) GRIN1-mediated activation of Cdc42 ↑ ($G\alpha_{i,o,z}$) cGMP-PDE ↑ ($G\alpha_t$) $G\alpha_{gust}$: ?	$G\alpha_{o1–2}$: neurons, neuroendocrine cells, astroglia, heart $G\alpha_{i1–i3}$: neurons and many others $G\alpha_z$: platelets, neurons, adrenal chromaffin cells, neurosecretory cells $G\alpha_{t1}$: rod outer segments, taste buds $G\alpha_{t2}$: cone outer segments $G\alpha_{gust}$: sweet and/or bitter taste buds, chemoreceptor cells in the airways	$G\alpha_{o(1/2)}$: PTX $G\alpha_{i1-i3}$: PTX $G\alpha_z$: ? $G\alpha_{t1/2}$: PTX, CTX $G\alpha_{gust}$: PTX
$G\alpha_{q/11}$	$G\alpha_q$ $G\alpha^{11}$ $G\alpha_{14}$ $G\alpha_{15}$ $G\alpha_{16}$	Phospholipase Cβ isoforms ↑ p63-RhoGEF ↑ ($G\alpha_{q/11}$) Bruton's tyrosine kinase ↑ ($G\alpha_q$) K^+ channels ↑ ($G\alpha_q$)	$G\alpha_{q/11}$: ubiquitous $G\alpha_{15/16}$: hematopoietic cells	$G\alpha_{q/11}$: YM-254890 $G\alpha_{14}$: ? $G\alpha_{15}$: ? $G\alpha_{16}$: ?
$G\alpha_{12/13}$	$G\alpha_{12}$ Gα13	Phospholipase D ↑ Phospholipase Ce ↑ NHE-1 ↑ iNOS ↑ E-cadherin-mediated cell adhesion: ↑ p115RhoGEF ↑ PDZ-RhoGEF ↑ Leukaemia-associated RhoGEF (LARG) ↑ Radixin ↑ Protein phosphatase 5 (PP5) ↑ AKAP110-mediated activation of PKA ↑ HSP90 ↑	Ubiquitous	$G\alpha_{12}$: ? $G\alpha_{13}$: ?
Gβ/γ	$\beta_{1–5}$ $\gamma_{1–12}$	PLCβs ↑ Adenylyl cyclase I ↓ Adenylyl cyclases II, IV, VII ↑ PI-3 kinases ↑ K^+ channels (GIRK1,2,4) ↑ Ca^{2+} (N-, P/Q-, R-type) channels ↓ P-Rex1 (guanine nucleotide exchange factor for the small GTPase Rac) ↑ c-Jun N-terminal kinase (JNK) ↑ Src kinases ↑ Tubulin GTPase activity ↑ G-protein-coupled receptor kinase recruitment to membrane ↑ Protein kinase D ↑ Bruton's tyrosine kinase ↑ p114-RhoGEF ↑	$\beta_1\gamma_1$: retinal rod cells $\beta_3\gamma_8$: retinal cone cells β_5: neurons and neuroendocrine organs $\beta_{5(L)}$: retina Most cell types express multiple β and γ subtypes	Gβγ: ?

CTX - cholera toxin; PTX - pertussis toxin; ↑ - enhances signal; ↓ - reduces signal; YM-254890 - a cyclic depsipeptide isolated from Chromobacterium sp QS3666

The first evidence of G-protein mediated signalling came in the 1970s, when several hormones were shown to modify the mode of cAMP production in cells and this production was found to be prolonged and increased by *Vibrio cholerae* toxin (Gill *et al.*, 1978). Later it was proposed that since the G-proteins are extremely conserved in different organisms and since several bacterial exotoxins act via covalent alteration of a G-protein α subunit, they could more easily overcome the species barrier. Finally, in 1980, the first G-protein was purified and recognized as a target for cholera toxin (Northup *et al.*, 1980). However, this was clearly not sufficient to completely explain the changes in cAMP levels after activation, because in some cases cAMP production was increased but in others it decreased. The answer came when *Bordetella pertussis* toxin was also recognised as a modifier of G-proteins (Bokoch *et al.*, 1984; Katada *et al.*, 1982). Two functionally distinct classes of Gα subunits were postulated, Gαs that stimulates and Gαi that inhibits cAMP production. Currently, many different Gα subunits are discovered and there are three major groups: Gαs and Gαi that act via adenylyl cyclase, the enzyme responsible for cAMP production; and Gαq that stimulates phospholipase Cβ (PLCβ), the enzyme that leads to intracellular Ca^{2+} mobilization (Milligan *et al.*, 2006).

In addition to the signal produced by Gα, the Gβ/γ dimer also interacts with signalling pathways and influences the ligand-induced response in the cell (Logothetis *et al.*, 1987). Intriguingly, in Gαq/Gαi knockout mice, overexpression of angiotensin II type I receptors leads to severe heart conditions that might be mediated by ERK signalling. This suggests that GPCR signalling is possible without the presence of a G protein (Rajagopal *et al.*, 2005; Zhai *et al.*, 2005).

In recent years, research has been focused on the "life cycle" of GPCRs, starting from their delivery to the cell membrane to internalisation following activation. Each step of receptor trafficking is crucial for functional activity and action of the receptor (Figure 1) (Drake *et al.*, 2006).

Similar to most cell membrane proteins, GPCRs are folded into the membrane during polypeptide chain synthesis at the endoplasmic reticulum (ER) and primed for delivery to their final destination by glycosylation (Alberts *et al.*, 2002). In the ER GPCRs undergo thorough quality control, followed by targeted degradation if misfolding or other errors in protein structure are discovered (Ellgaard *et al.*, 2003).

Protein chaperones are also reported to facilitate or detain delivery of GPCRs to the cell membrane (Chapple *et al.*, 2003).

After GPCRs are trafficked to plasma membrane, several factors can affect their maintenance and signalling ability. First, their localization within the membrane specificially in lipid rafts or calveolae (regions of cell membrane abundant with glycosphingolipids, cholesterol and different proteins), can influence GPCR signalling (Chini *et al.*, 2004). Characteristics of the membrane surrounding GPCR can attract distinct types of Gα subunits or colocalization of GPCRs and adenylyl cyclase in specific lipid compartments can enhance signalling efficacy (Oh *et al.*, 2001; Ostrom *et al.*, 2001). Several proteins are important in receptor stabilization and maintenance in the plasma membrane as well (Tan *et al.*, 2004; Xiang *et al.*, 2002).

The standard view of GPCR function is that they are present as monomeric receptors in the cell membrane, but early studies have already demonstrated possible formation of oligomers. Many GPCR clearly require formation of homodimeric or heterodimeric aggregates to function, therefore, the study of oligomerization is a popular area of GPCR research (Drake *et al.*, 2006; Gurevich *et al.*, 2008; Park *et al.*, 2004).

The first convincing evidence of GPCR aggregates came with the study of γ-aminobutyric acid b receptors (GABAb) showing that heterodimerization of two receptors, GABAb-R1 and GABAb-R2, in the ER is essential for receptor transport to the plasma membrane (Marshall *et al.*, 1999). Further research revealed that this oligomerization is also crucial for receptor activity (Margeta-Mitrovic *et al.*, 2000). Functional studies on other receptors demonstrated oligomerization in the ER, but the function of this aggregation requires further investigation (Floyd *et al.*, 2003; Issafras *et al.*, 2002). GPCRs are also reported to form oligomers in the plasma membrane after activation by agonist (Kroeger *et al.*, 2001; Wurch *et al.*, 2001); however, ligand-induced inhibition of oligomer formation has also been observed (Cheng *et al.*, 2001; Latif *et al.*, 2002).

Oligomerization may change the functional characteristics of GPCR and alter ligand-binding profiles (Suzuki *et al.*, 2006; Yoshioka *et al.*, 2001). For example, the heteromerization between chemokine receptors CCR2 and CCR5, can lead to increased response to the chemokine signal and even transduction of the signal via a Gαq pathway that is not typical for each receptor acting separately (Mellado *et al.*,

2001). The role of GPCR oligomers in internalization and desensitization processes has also been explored (Ecke *et al.*, 2008; Stanasila *et al.*, 2003). Many receptor oligomers internalize upon activation of only one receptor ligand of the pair, while some receptors detain endocytosis of others by formation of heteromers (Hillion *et al.*, 2002; Lavoie *et al.*, 2002). Nonetheless, oligomerization of GPCRs can strongly influence the "life cycle" and signalling ability of the receptors and further studies will discover the practical application of these results.

Following activation, GPCRs undergo desensitization and internalization. First, after agonist activation, serine and threonine residues of the GPCR on the intracellular side of the receptor are phosphorylated by GPCR kinases (GRKs) (Ribas *et al.*, 2007). The phosphorylated residues attract and bind β-arrestin, which further forms a complex with activating protein 2 (AP-2) and clathrin, leading to internalization of GPCR in clathrin-coated vesicles (Attramadal *et al.*, 1992; Goodman *et al.*, 1996; Laporte *et al.*, 1999). In the intracellular space, internalized GPCRs can either be recycled back to the cell surface via the sorting machinery that recognizes specific structural signals (Barak *et al.*, 1994; Schulein *et al.*, 1998) or be degraded by classical ubiquitination (Wojcikiewicz, 2004). However, several reports have demonstrated that β-arrestin is involved not only in the internalization process, but also in GPCR ubiquitination, suggesting β-arrestin dependent degradation (Martin *et al.*, 2003; Shenoy *et al.*, 2005).

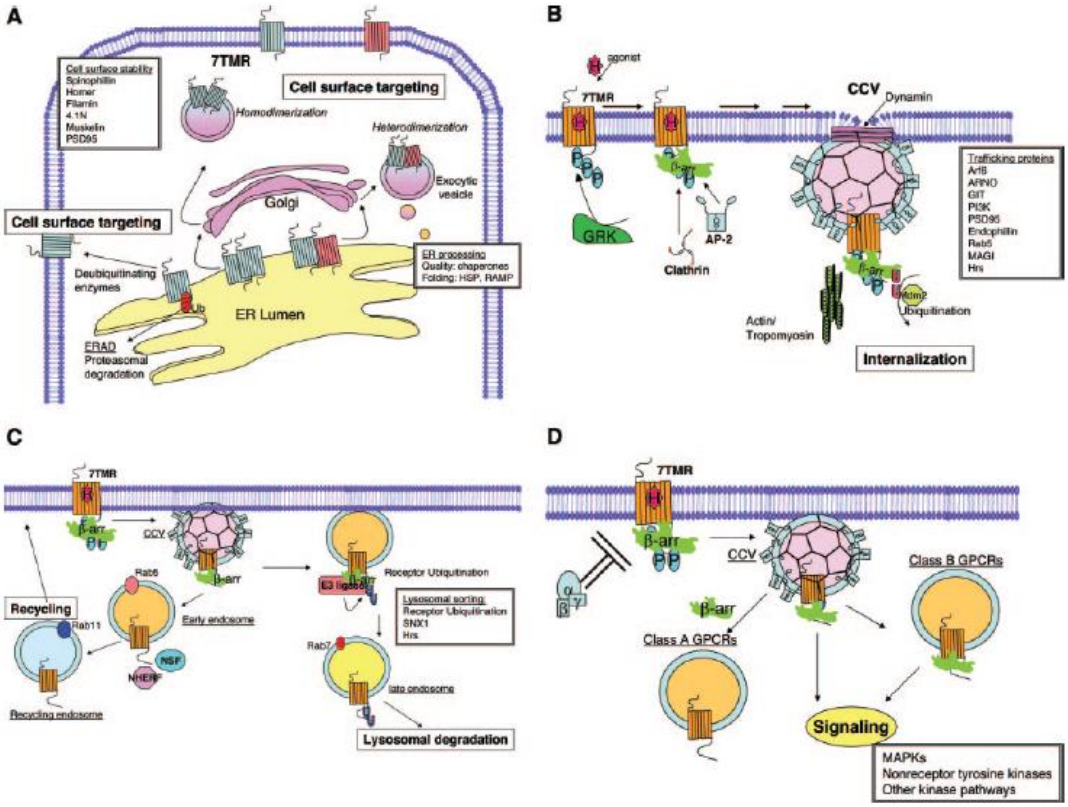

Figure 1. "Life cycle" of GPCRs. A Synthesis and trafficking to plasma membrane. B Internalization. C Degradation or recycling. D Signalling via β-arrestin (Figure is from Drake et al. 2006).

1.1.3 Functional expression of GPCR in yeast

The expression of GPCRs in yeast has specific advantages over mammalian cell model systems. The signalling systems of GPCRs in yeast are very similar to mammalian and yeast have only two endogenous GPCR pathways, both of which can be switched off by elimination of gene expression or knocked out by deletion from the yeast genome, by trivial technical manipulations that are much more complicated in mammalian experiments. Yeast grow faster and maintenance is more simple than for mammalian cells. Furthermore, yeast systems can be automated for high-throughput screening. The drawback of receptor expression in yeast is that proteins in yeast have different post-translational modifications than in mammalian cells, which might affect trafficking of the receptors to the cell surface. Another disadvantage is the dense polysaccharide rich cell wall of yeast that can detain ligand diffusion to the receptor (Dohlman *et al.*, 1991; Ladds *et al.*, 2005; Pausch, 1997).

Saccharomyces cerevisiae has two native GPCR signalling systems: pheromone and glucose pathways. The glucose pathway induces active cell metabolism and proliferation while the pheromone signalling pathway ensures specific physiological alterations in haploid cells that are crucial for mating and formation of diploid cells (Beullens *et al.*, 1988; Donzeau *et al.*, 1999). The glucose pathway in yeast is vital for functional maintenance and propagation. Therefore, GPCR research in *Saccharomyces cerevisiae* uses a modified pheromone signalling pathway for receptor expression and activation detection (Versele *et al.*, 2001).

The natural yeast pheromone pathway starts when the pheromone signalling molecule binds to the receptor and activated G protein diffuses from the receptor into the cellular space. The G protein divides in Gα (Gpa1p) and Gβ/γ (Ste4p/Ste18p) dimer and a complex of Gβ/γ activates the protein kinase Ste20p, which triggers the MAP kinase cascade. The MAP kinase cascade causes functional changes in yeast haploid cells that lead to the mating and fusion of two haploid cells into a diploid (Leberer *et al.*, 1992; Whiteway *et al.*, 1989). These changes include Far1 inhibitor activation enabling cell-cycle arrest and stimulation of Ste12p that leads to expression of mating specific genes, for example, the adhesion factor Fus1(Chang *et al.*, 1990; Nomoto *et al.*, 1990; Stevenson *et al.*, 1992). Another yeast regulatory protein, Sst2, activates GTPase and triggers Gα inactivation, thus controlling mating capacity (Dohlman *et al.*, 1996).

Yeast strains have been generated for expression of heterologous GPCRs (Cismowski *et al.*, 1999; Kajkowski *et al.*, 1997; Price *et al.*, 1996) by altering the natural yeast mating signalling system. In these modified yeast strains, the genes for the pheromone receptors Ste2 and Ste3 are deleted to exclude possible competition for G proteins between natural yeast mating receptors and the transformed heterologous receptors. The native Gα subunit gene (Gpa1p) is substituted with a chimeric Gα, one part of which represents a human Gα protein to ensure binding to the receptor, and other derived from yeast Gα protein for binding of the Gβ/γ dimer (Brown *et al.*, 2000; Kang *et al.*, 1990). The regulatory protein Sst2 is inactivated, so the Gα subunit can remain in active state longer, resulting in more sensitive receptor activity detection (Garrison *et al.*, 1999). The gene for Far1 is also deleted so yeast cells can continue to proliferate after activation of the MAP kinase cascade (Chang *et al.*, 1990). Finally, to generate a quantitative signal of receptor activity, the promoter of

the gene *Fus1* is fused with reporter genes *His3* and *lacZ*. Gene *His3* encodes an enzyme (imidazole acetol phosphate transaminase) crucial for histidine production. Receptor activation leading to *His3* expression enables yeast to grow on histidine-deficient medium, while *lacZ* provides expression of β-galactosidase that cleaves a substrate for measurement of a colour change (Nomoto et al., 1990; Stevenson et al., 1992). Natural and modified yeast pheromone signalling pathways are in Figure 2. However, the reporter gene *His3* has background activity in minimal medium lacking histidine, so addition of 3-amino-1,2,4-triazole (AT) is necessary to improve sensitivity of the method (Cismowski *et al.*, 1999).

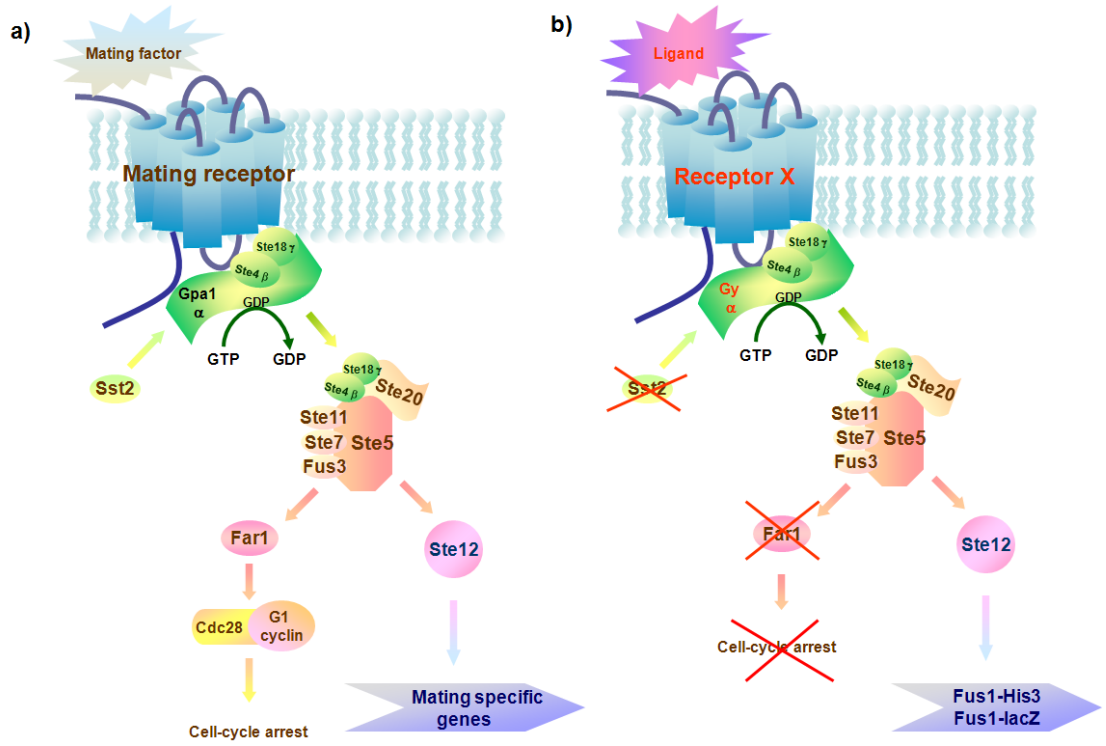

Figure 2. a) Natural and b) modified yeast pheromone signalling pathway (Figure created by the author of the thesis, based on descriptions in literature).

Other modifications vary depending on the specific characteristics of the receptor. To increase trafficking efficiency to the cell surface, the receptor can be fussed to a leader sequence. Better expression of some GPCRs can be achieved by deletion or substitution of specific regions of the receptor, modifying or regulatory proteins can also optimise the system (Erlenbach *et al.*, 2001; Miret *et al.*, 2002).

Research on GPCRs in *Saccharomyces cerevisiae* has different approaches. For example, regions of the receptors involved in the receptor activation process can be detected by expression of mutant receptor libraries in yeasts. Novel peptide agonists can be identified by screening mutant peptide libraries by co-expression with a target receptor (Geva *et al.*, 2000; Sachpatzidis *et al.*, 2003). Orphan receptors can be tested by high-throughput screening with ligand libraries to discover possible agonists and "deorphanise" the receptor (Brown *et al.*, 2003). Yeast can also be used to study proteins that regulate G protein activity (Cao *et al.*, 2004) or enzymes that phosphorylate GPCRs (Noble *et al.*, 2003). Heteromerization with other proteins that affect ligand specificity can also be explored (Miret *et al.*, 2002).

A revolutionary approach to GPCR mutagenesis in yeast started with attempts to isolate receptors activated solely by synthetic ligands (RASSL) or designer receptors exclusively activated by a designer drug (DREADD) (Coward *et al.*, 1998; Dong *et al.*, 2009). Both of these techniques focused on detection of receptor forms whose activity could be induced in either knockout or wild-type animals to target specific tissues for deeper comprehension of functional regulation (Sweger *et al.*, 2007). In this manner, variants of randomly mutagenized rat muscaric receptor 3 were isolated and their activation by a synthetic ligand (clopazine-N-oxide), but not the natural agonist acetylcholine was detected (Armbruster *et al.*, 2007).

Yeast have also been used for research on GPCR oligomerization. Floyed and colleagues used a fluorescence resonance energy transfer (FRET) method to show that CCR5a forms oligomers in an *S.cerevisiae* expression system (Floyd *et al.*, 2003).

In addition to *S.cerevisiae*, other yeast species can be used for GPCR research. For example, Ladds et al. have expressed corticotrophin-releasing factor receptors in *Schizosaccharomyces pomb*e and showed that diverse ligands can affect receptor conformational changes and lead to the binding of different G protein subunits in the intracellular space (Ladds *et al.*, 2003).

1.2 Proopiomelanocortin signalling

Melanocortin receptors (MCRs) are a distinct group of GPCRs. All MCRs are activated by melanocortins that are neurogenic peptides derived from a single precursor, proopiomelanocortin (POMC). POMC is expressed in various tissues,

predominantly in the pituitary and areas of the brain and skin, where it undergoes enzymatic cleavage into several signalling peptides (Figure 3). Cleavage of POMC is tissue specific, leading to different signalling peptide profiles in different tissues. Signalling peptides that act via MCRs are α-melanocyte stimulating hormone (MSH), βMSH, γMSH and adrenocorticotropic hormone (ACTH) (Crine *et al.*, 1979; Hadley *et al.*, 1999; Mains *et al.*, 1979). After production in the pituitary, both αMSH and ACTH are delivered to their site of action in an endocrine manner via blood circulation. However, since MCRs are abundantly expressed in the central nervous system (CNS), POMC peptides can signal in a paracrine way to surrounding tissues as well (Hadley *et al.*, 1999). Two other peptides that are also expressed in the CNS act as natural antagonists of MCRs: agouti and agouti-related protein (AGRP) (Graham *et al.*, 1997; Lu *et al.*, 1994).

Five different MCRs have been identified in humans: MC1R, MC2R, MC3R, MC4R and MC5R (Chhajlani *et al.*, 1992; Gantz *et al.*, 1993; Gantz *et al.*, 1994; Mountjoy *et al.*, 1992; Roselli-Rehfuss *et al.*, 1993). A detailed overview of MCRs is in Table 3.

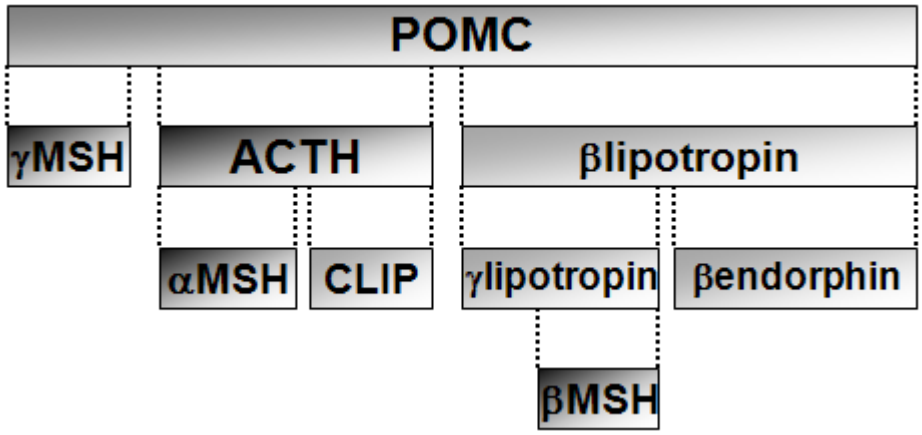

Figure 3. Peptides that are derived from proopiomelanocortin during post-translational processing (Figure created by the author of the thesis, based on descriptions in literature).

Table 3. Subtypes, chromosome position, pharmacological profiles, functions and sites of expression of melanocortin receptors (Table summarized by the author of the thesis from IUPHAR database (http://www.iuphar-db.org) and literature used in thesis)

Receptor	Gene localization	Natural agonist	**Full agonist**/partial agonist affinities	**Antagonist**/inverse agonist affinities	Signal transduction mechanism	Tissue expression	Functions
MC1R	16q24.3	αMSH	**[125I]NDP-MSH** >SHU9119 >HS014 **>NDP-MSH >α-MSH**	**agouti >HS024 >ASIP [90-132 (L89Y)]**	Gαs	Melanocytes, neutrophils, endothelial cells, dermal papilla cells, pituitary and testes	Pigmentation (synthesis of eumelanin), anti-inflammatory actions
MC2R	18p11.21	ACTH	ACTH (1-39) > **ACTH (1-24)**	**ACTH (11-24)** > ASIP [90-132 (L89Y)]	Gαs	Adrenal gland, adrenal cortex, skin tissue	Adrenal cortical steroidogenesis
MC3R	20q13.2	γMSH	**[125I]NDP-MSH > γ-MSH > NDP-MSH > MT-II > α-MSH > D-Trp8 γ-MSH**	**HS024 > HS014 > SHU9119** > AGRP > agouti	Gαs	Stomach, duodenum and pancreas, heart, placenta, CNS, kidneys	Energy homeostasis, naturesis
MC4R	18q21.3	αMSH	**[125I]NDP-MSH > NDP-MSH > MT-II > α-MSH > THIQ > RY764**	**MCL0129 > HS014 > SHU9119 > MBP10** > AGRP	Gαs	Various areas of the CNS, dermal papilla cells	Feeding behaviour, the regulation of metabolism, sexual behaviour, male erectile function
MC5R	18p11.2	αMSH	SHU9119 > **[125I]NDP-MSH > α-MSH > PG-901 > NDP-MSH**	**HS024** > agouti > AGRP	Gαs	Adrenal gland, fat cells, kidney, leukocytes, lung, lymph node, mammary gland, ovary, pituitary, testis, uterus, various exocrine cells	Water and thermoregulation, production of sebaceous lipids, sexual behaviour

1.2.1 MC4R

The cloning of MC4R and characterization of its expression profile was performed in the early 1990s. The primary sites of MC4R expression are the ventromedial, dorsomedial, paraventricular and arcuate hypothalamic nuclei regions of the CNS. The natural agonist of MC4R is αMSH, but with lower affinity also βMSH and γMSH. Agouti and agouti-related protein (AGRP) are antagonists of MC4R (Gantz *et al.*, 1993; Mountjoy *et al.*, 1994).

Observations of the possible physiological functions of MC4R began long before the discovery of the receptor itself. First, a relationship between rodent coat colour and obesity was noticed along with a change of hair colour upon αMSH injection (Geschwind, 1966). Later, αMSH was found to stimulate both MC1R (responsible for pigmentation) and MC4R (responsible for feeding behaviour). Mice with light hair colour and obesity phenotypes were found to be carriers of genetic mutations in the agouti gene (Miller *et al.*, 1993). The most convincing evidence of role of MC4R in feeding behaviour came with phenotype characterization of MC4R knockout mice. MC4R knockouts demonstrated obese, hyperphagic and hyperinsulinemic phenotypes (Huszar *et al.*, 1997).

The mechanism of MC4R action is complex and it integrates of various feeding signals that are reviewed and processed in the arcuate nucleus of the hypothalamus including leptin, cocaine-amphetamine-regulated transcripts, ghrelin and other systems. The signal downstream of MC4R is further processed in the ventromedial and lateral hypothalamus nuclei where it is integrated with orexin, the melanin-concentrating hormone system and other pathways. All these signalling routes interact with each other and are regulated by different feedback systems, both within and outside the hypothalamus (Cone, 2006).

Another important regulator of MC4R-mediated signalling is mahogany protein. The mahogany protein is reported to be a TM attractin that is widely expressed and is possibly involved in low-affinity agouti binding. The mahogany protein directly interacts with MC4R, but further research is needed to explain the underlying mechanisms (Gunn *et al.*, 1999; Haqq *et al.*, 2003; Nagle *et al.*, 1999).

MC4R has been reported to form homooligomeric structures, and heterooligomerization between MC4R and the kappa opioid receptor has been

investigated; however, the interaction signal is at the detection threshold and the possible physiological role of dimerization is unclear (Nickolls *et al.*, 2006).

Genetic polymorphisms and mutations in *MC4R* are mainly associated with obesity-related phenotypes. This is further discussed in Chapter 1.2.4, Genetic studies of *MC4R*.

1.2.2 Other melanocortin receptors

MC1R has wide tissue expression, and is detected in melonocytes, pituitary, fibroblasts, and immune cells (Mountjoy *et al.*, 1992). The natural agonist of MC1R is αMSH and its antagonist is agouti. The main physiological function of MC1R is considered to be pigmentation of skin and hair. After receptor stimulation, Gαs activates adenylyl cyclase, which increases the intracellular cAMP concentration, triggering eumelanin synthesis leading to the formation of dark pigment (Rana, 2003). Homodimerization of MC1R has been reported by several research groups (Mandrika *et al.*, 2005; Sanchez-Laorden *et al.*, 2006; Zanna *et al.*, 2008). Intriguingly, dimerization has also been described between wild type MC1R and several polymorphic MC1R variants associated with pigmentation. Furthermore, this oligomerization process has been demonstrated to influence receptor signal transduction (Sanchez-Laorden *et al.*, 2006).

MC2R is primarily expressed in the adrenal cortex where it regulates steroidogenesis. For a long time, attempts to functionally express MC2R were unsuccessful, delaying investigation of this receptor. Expression of recombinant MC2R was eventually achieved in adrenal cell lines (Schimmer *et al.*, 1995). Intriguingly, the use of non-adrenal cell lines was unsuitable because in all other cells, MC2R was detained in the ER and not transported to the cell membrane (Webb *et al.*, 2009). This led to the suggestion that adrenal cells contain a specific signalling mechanism to direct MC2R to the membrane, leading to the discovery of melanocortin receptor accessory protein (MRAP). Furthermore, co-expression of MRAP and MC2R in non-adrenal origin cells enabled MC2R transport to the cell membrane (Metherell *et al.*, 2005).

The highest MC3R expression levels are detected in the ventromedial hypothalamus, medial habenula, ventral tegmental area and raphe (Roselli-Rehfuss *et al.*, 1993). MC3R is involved in regulation of different autonomous functions and

inflammatory responses. The distinction between MC3R and other MCRs is that beside signal transduction via Gαs, MC3R has also been reported to couple to Gαq (Konda *et al.*, 1994). MC3R is demonstrated to form homooligomers as well as heteromers with MC1R (Mandrika *et al.*, 2005). Interestingly, MC3R heterooligomerization with ghrelin receptor affects ghrelin signalling, implicating this receptor in feeding behaviour (Rediger *et al.*).

MC5R is mainly expressed in periphery tissues: skin, muscles and exocrine glands. The functions of this receptor remained unclear until the development of the knockout mouse. At first, no specific phenotypic features of knockouts were observed. However, after a swim test, MC5R-deficient mice retained wet coats for longer periods than wild type. This led to the discovery that MC5R regulates secretion in exocrine glands (Chen *et al.*, 1997). Recently, MC5R has also been implicated in regulation of sexual behaviour (Morgan *et al.*, 2006). In addition to MC2R, MC5R has been reported to interact with MRAP. Contrary to MC2R, MRAP is responsible for detaining MC5R cell surface trafficking and blocking homooligomerization (Sebag *et al.*, 2009).

1.2.3 Functional regions of MC4R

Since MC4R is a key player in feeding behaviour, the main pharmacological interest of many MC4R studies is developing new adiposity treatment drugs that act via MC4R. Targeted design of novel ligands requires insight into the regions of MC4R that are important for functional activity. Several studies have explored the MC4R ligand-receptor interaction and signal transduction mechanisms (Fleck *et al.*, 2005; Nickolls *et al.*, 2003; Yang *et al.*, 2000).

The characteristic amino acid sequence His-Phe-Arg-Trp of αMSH is a pharmacophore that is the amino acid sequence of the peptide that interacts with the MC4R binding pocket. The pharmacophore of AGRP is reported to be Arg-Phe-Phe. The binding pocket of MC4R that interacts with the pharmacophore of its natural ligand αMSH is considered to have two distinct parts - a negatively charged conformation pocket and a hydrophobic cavity (Wilczynski *et al.*, 2004). A possible model of MC4R binding with αMSH and AGRP pharmacophores is in Figure 4.

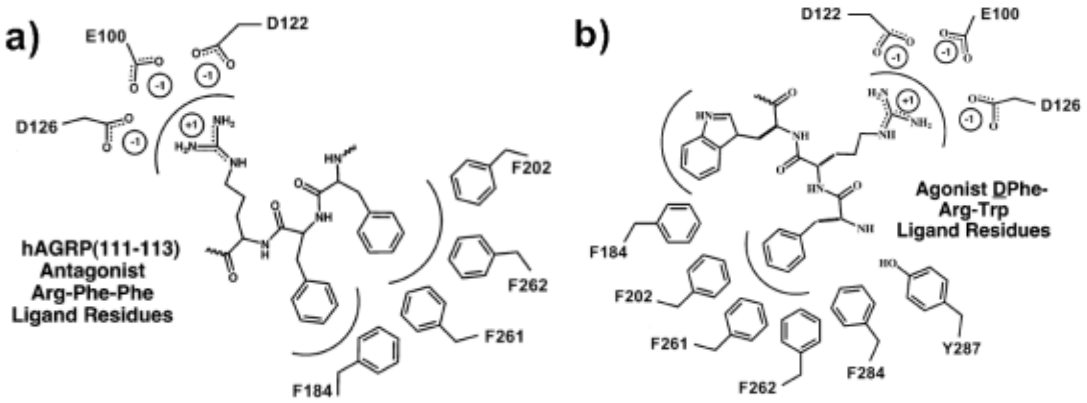

Figure 4. Schematic representation of a) natural antagonist pharmacophore and b) natural agonist pharmacophore binding pocket of MC4R (adapted from Wilczynski et al. 2004).

A functional mutagenesis study by Yang and colleagues showed that the single substitutions E61A, D90A, E100A, D122A, D122N, M200A, M204A, W258A, F261A, D298A, R305A in the MC4R significantly decrease receptor binding affinity for αMSH and NDP-MSH, but substitutions D126A, D126N or double substitutions D122N/D126N or D122A/D126A completely abolish αMSH binding to MC4R and have a significant effect on binding of NDP-MSH and AGRP. Overall, these data also correlate with the changes in cAMP half-maximal effective concentration (EC_{50}) values (Yang *et al.*, 2000). The positions E100, D122, D126, F254, W258, F261 and H264 are also involved in THIQ binding and receptor activation (Yang *et al.*, 2009).

D122A substitution was later demonstrated to result in significantly lower binding affinities for other peptides and non-peptide agonists and antagonists. D126A, M200A, F261A and F284A mutant receptors are important in ligand binding in similar studies (Fleck *et al.*, 2005; Nickolls *et al.*, 2003). Additionally, I125F and I137T MC4R variants have significantly decreased affinity for THIQ and SHU9119 (Nickolls *et al.*, 2003). The binding properties of the MC4R antagonist SHU9119 have been explored and, interestingly, L133M substitution leads to an MC4R signalling swap from antagonist to agonist (Yang *et al.*, 2002). A slight influence of amino acids in positions 125, 129, 268, 287, 291 on ligand binding to MC4R has also been observed (Hogan *et al.*, 2006). A functional study of mouse MC4R confirmed the human receptor data for positions E100, D122 and D126 (with corresponding residues in mouse MC4R: E92, D114, D116), but F184 (F176 in mouse) significantly influenced binding of NDP-MSH, MTII and AGRP (Haskell-Luevano *et al.*, 2001); in human MC4R, substitution to Ala causes no significant effects (Hogan *et al.*, 2006). Inconsistent results were also observed for residue F261; the human F261A MC4R

variant has significantly decreased affinity for NDP-MSH, but not for AGRP while the corresponding F253A variant of the mouse receptor shows significantly lower affinity for AGRP and not for NDP-MSH (Haskell-Luevano *et al.*, 2001; Yang *et al.*, 2000).

Several MC4R residues have been mutagenised to determine G-protein coupling characteristics in the intracellular part of the receptor. The results highlighted R220A, T232A, T232V mutant receptors as having significantly decreased signalling efficacy (Kim *et al.*, 2002). Mutagenised residues of the MC4R are in Figure 5.

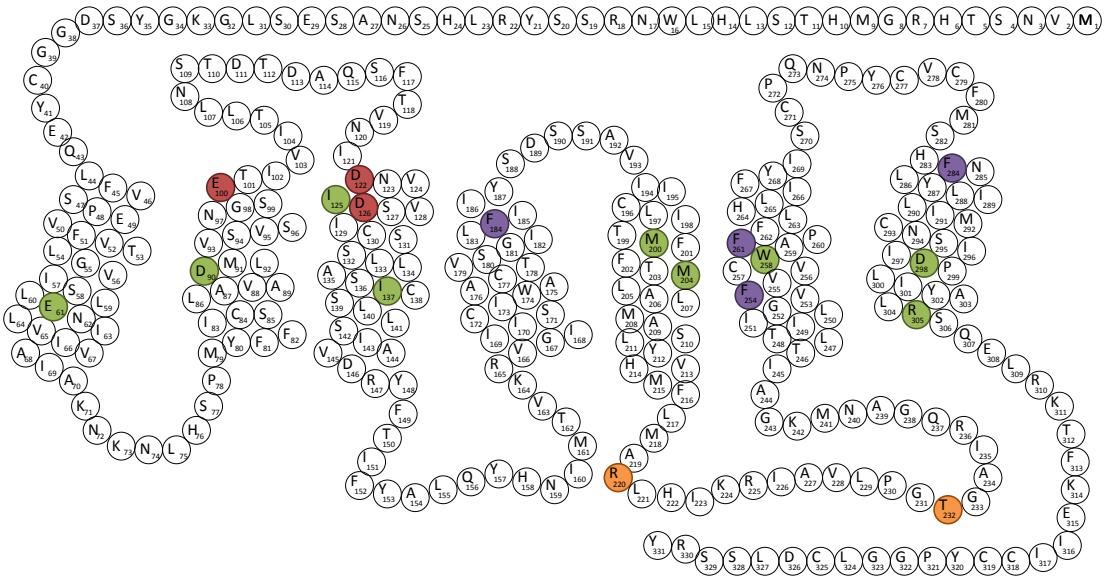

Figure 5. Schematic diagram of MC4R. Coloured amino acids are reported to be important using mutagenesis. Highlighted in red are amino acids, that are believed to be involved in forming of acidic pocket, in violet are amino acids, that are believed to form hydrophobic pocket, in green – other residues responsible for receptor activation, in orange – residues involved in G protein coupling (Figure created by the author of the thesis, based on descriptions in literature).

1.2.4 Genetic studies of *MC4R*

The first evidence on the influence of genetic variants on human obesity-related traits came from multiple studies analysing polymorphisms/mutations and deletions in coding region of *MC4R*. Generally, the reports analysed different cohorts with distinct obesity phenotypes such as severe obesity or early childhood obesity. A detailed map of the most common genetic variants discovered in *MC4R* is in Figure 6. Alongside variation detection, research on these positions usually involves functional characterization of the found alterations. One of the most common nonsynonymous substitutions in the *MC4R* coding region is V103I (rs code: rs2229616, MAF =

0.016). Some studies pointed out that this variant has a somewhat protective effect against obesity, as demonstrated by genetic association studies (Stutzmann *et al.*, 2007; Wang *et al.*, 2010). However, functional analysis of mutant MC4R containing the V103I substitution demonstrated that this alteration does not affect trafficking or signalling ability of the receptor when activated by agonist (Hinney *et al.*, 2003; Ho *et al.*, 1999), but it has also been shown that V103I receptor variant has decreased AGRP binding (Xiang *et al.*, 2006). Alterations reported to have an impact on MC4R trafficking are D37V (rs13447325), P78L (rs13447326), R165W (rs13447332), P299H (rs52804924), and I317T (rs13447337). Alterations P78L (rs13447326), S94R (rs13447327), V95I (rs13447328), I121T (rs13447330), S127L (rs13447331), G181D (rs13447333), P230L (rs13447334), and A244E (rs13447335) have significant decrease signalling transduction. Genetic variants T112M (rs13447329), I169S (rs1016862), and I251L (rs52820871) do not seem to affect receptor function (Calton *et al.*, 2009; Fan *et al.*, 2009; Hinney *et al.*, 2003; Lubrano-Berthelier *et al.*, 2003; Valli-Jaakola *et al.*, 2004; Xiang *et al.*, 2006). Interestingly, alteration D90N (no rs code) is reported to have an autosomal dominant effect in heterozygous carrier. This is explained by oligomerisation between MC4R expressed from the wild type allele and the mutant D90N MC4R that affect the signal transduction mechanisms of the receptor (Biebermann *et al.*, 2003).

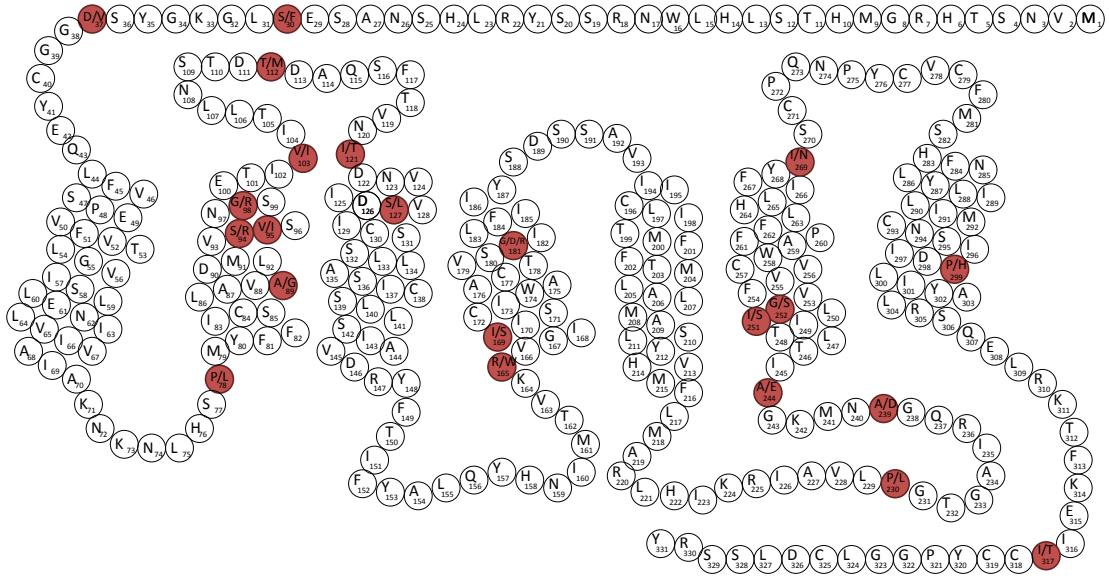

Figure 6. Schematic diagram of MC4R. Highlighted in red are nonsynonymous coding amino acid substitutions described in literature, also frameshift coding deletions in positions L322, I251,

L211 and deletion in position Y35 leading to stop codon has been reported (Figure created by the author of the thesis, based on data from Ensemble Genome Browser, www.ensembl.org).

The first important report that associated common polymorphisms in the *MC4R* region with obesity was a meta-analysis of several genome-wide association studies (GWAS) (Consortium, 2007). The main goal of this research was to identify single-nucleotide polymorphisms (SNPs) associated with body mass index (BMI). A very strong association was found with rs17782313 located upstream of *MC4R* in an initial scan of over 16,000 people; the authors also generalised these findings in other populations as well (Loos *et al.*, 2008). This study was replicated in other cohorts to find associations between rs17782313 and other SNPs in the *MC4R* region with BMI and obesity-related phenotypic traits. rs17782313 was found to be associated with BMI, waist circumference, body weight, hypertension, some characteristics of body fat distribution and other phenotypes (Hardy *et al.*, 2010; Haupt *et al.*, 2009; Timpson *et al.*, 2009; Zobel *et al.*, 2009).

Another SNP that is frequently genotyped in the *MC4R* locus is rs12970134. There is a high linkage disequilibrium (LD) between rs12970134 and rs17782313 (r^2 = 0.8) suggesting that rs12970134 should also be associated with BMI-related conditions. However, results on rs12970134 are not consistent, although evidence suggests that rs12970134 is associated with BMI, waist circumference and insulin resistance (Been *et al.*, 2009; Chambers *et al.*, 2008; Zobel *et al.*, 2009). Another study has demonstrated no association with any BMI-related phenotypes, however (Ng *et al.*, 2010).

Several other SNPs including rs477181, rs502933, rs4450508 in the *MC4R* locus are not associated with any BMI condition (Been *et al.*, 2009; Zobel *et al.*, 2009), but another report showed association with waist circumference (Chambers *et al.*, 2008). rs571312, rs10871777 and rs476828 are in strong LD (r^2 = 1) with rs17782313 and associated with obesity (Grant *et al.*, 2009).

1.3 FTO

In addition to MC4R, the FTO (fat mass and obesity-associated protein) is an important regulator of body mass and energy homeostasis. The first evidence of *FTO* polymorphism correlation with weight-related phenotypes came from GWAS performed in a Finnish population that identified a genetic variant in the *FTO* locus as

a SNPs associated with T2D. However, upon adjustment for BMI, this association weakened (Scott *et al.*, 2007). Another GWAS study found significant association of rs9939609 located in the first intron of *FTO* with increased BMI (Frayling *et al.*, 2007). These results were replicated in multiple populations where the *FTO* locus was been associated with various BMI and obesity-related phenotypes such as hip and waist circumference, weight, T2D, higher energy intake and decreased satiety (Cecil *et al.*, 2008; Frayling *et al.*, 2007; Scott *et al.*, 2007; Scuteri *et al.*, 2007; Wardle *et al.*, 2008).

After implication of the genetic variants of the *FTO* in body mass regulation, studies of the physiological functions of *FTO* encoded protein began. The functional domains of the FTO are related to enzyme group – Fe(II)- and 2-oxoglutarate–dependent oxygenases. Furthermore, FTO is demonstrated to participate in DNA demethylation (Gerken *et al.*, 2007). Homologous sequences to the *FTO* locus have been found in organisms from algae to humans, indicating the ancient origin of the gene (Fredriksson *et al.*, 2008). *FTO* is ubiquitously expressed, with the highest levels in brain and hypothalamus, the regulation centre of feeding behaviour (Frayling *et al.*, 2007; Fredriksson *et al.*, 2008). Upregulation of *FTO* is observed in rats after 48 h food restriction (Fredriksson *et al.*, 2008). However, decreased *FTO* expression is observed in obese mice (mice strains A^{y}, Lep^{ob}, $Lepr^{db}$, Cpe^{fat}, *tub*) upon food deprivation (Stratigopoulos *et al.*, 2008). Two recombinant FTO mice models have been phenotypically characterized. First, model mice with an introduced I367A mutation in the sixth exon of *FTO*. It has been showed that *in-vitro* this alteration leads to decreased dimerization of FTO that is essential for full catalytic activity of the enzyme. Mice containing a I367A substitution demonstrated reduced body weight (Church *et al.*, 2009). The second mouse model is deficient for FTO and has decreased fat mass and growth retardation (Fischer *et al.*, 2009).

Strong association of *FTO* with obesity is assumed to be due to linkage disequilibrium with other functional SNPs or associated genetic variants localized in regions responsible for regulation of other genes (Frayling *et al.*, 2007). However, functional studies indicate that FTO has physiological functions in body mass regulation (Church *et al.*, 2009).

1.4 Purinergic receptors

Signalling by purines in different tissues has been reported since the mid-20th century (Forrester *et al.*, 1969; Mills *et al.*, 1968). In the 1970s and 1980s the two groups of purine-recognizing receptors were described, dividing the adenosine and adenosine triphosphate (ATP)/adenosine diphosphate (ADP) receptors (Spedding *et al.*, 1976). The next step in classification of purinergic receptors was the establishment of two receptor types: P2XRs (ion channels) and P2YRs (GPCRs) (Abbracchio *et al.*, 1994). However, the cloning and pharmacological characterization of P2YRs started only in the beginning of 1990s due to technology development. P2Y1R was first cloned and recognised to bind ADP (Lustig *et al.*, 1996; Webb *et al.*, 1993) followed by all other receptors in this group: P2Y2R, P2Y4R, P2Y6R, P2Y11R, P2Y12R, P2Y13R and P2Y14R (Chambers *et al.*, 2000; Communi *et al.*, 1997; Communi *et al.*, 1996; Communi *et al.*, 1995; Hollopeter *et al.*, 2001; Janssens *et al.*, 1996).

Currently, eight P2YRs are described in humans. All missing numbers in the classification order (P2Y3R, P2Y5R, P2Y7R, P2Y8R, P2Y9R, P2Y10R) constitute P2YR that are expressed in other organisms or are still orphan receptors that share sequence similarity to P2YR. The natural ligand spectrum of P2YR receptors is ATP, ADP, uridine triphosphate (UTP), uridine diphosphate (UDP) and UDP-glucose. All transduce signals via Gαq or Gαi of Gα subunits and all except P2Y11R are coded for intronless genes (Abbracchio *et al.*, 2006). A detailed overview on P2YRs is in Table 4.

Table 4. Subtypes, chromosome position, pharmacological profiles, functions and sites of expression of purinergic receptors (Table summarized by the author of the thesis from IUPHAR database (http://www.iuphar-db.org) and literature used in thesis)

Receptor	Gene localization	Natural agonist	**Full agonist**/partial agonist affinities	Antagonist affinities	Signal transduction mechanism	Tissue expression	Functions
P2Y1R	3q24–25	ADP	2',3'-ddATP >dATPαS >ATPγS > 2MeSATP >ATP >**ADP** >**2MeSADP**	[32P]MRS2500 >[3H]MRS2279 >MRS2500 >suramin >A2P5P	Gαq	Blood platelets, placenta, brain, intestine, skeletal muscle, heart, macrophages, pituitary, lung, pancreas, spleen, adipose, stomach, lymphocytes, liver, kidney	Induction of platelet aggregation and cell shape change, endothelial cell migration, regulation of epidermal growth factor activity
P2Y2R	11q13.5	ATP=UTP	**MRS2698 =UTP =ATP >Up4U>UTPγS>5BrUTP**	AR-C126313 >=Reactive Blue-2 >suramin	Gαq	Brain, bronchial epithelium, epidermal cells	Control of cellular proliferation, spreading and migration, ion transport control, chloride secretory response
P2Y4R	Xq13	UTP	**(N)methanocarba-UTP >Up4U>UTP** >ATP>GTP >CTP	ATP >PPADS >Reactive Blue-2	Gαq	Intestine, brain , adipose, lung, skeletal muscle, spleen, lymphocytes, prostate, heart, pancreas, placenta, kidney, stomach, bone, bronchial epithelium	Regulation of chloride epithelial transport, ion transport control
P2Y6R	11q13.5	UDP	**MRS2693 >UDP-β-S >Up3U >UDP** >UTP >ADP >2MeSATP	MRS2578 >MRS2567 >MRS2567 >Reactive Blue-2	Gαq	Spleen, placenta, kidney, intestine, adipose, bone, lung, heart, brain, skeletal muscle, stomach, bronchial epithelium	Contraction of smooth muscle, ion transport control, anti-inflammatory actions
P2Y11R	19p31	ATP	**AR-C67085>ATPγS >UTP >dATP >ATP >2MeSATP >NAD**	NF157 >NF340 >suramin >Reactive Blue-2	Gαq	Platelets, brain, pituitary, lymphocytes, spleen, intestine, macrophages, lung, stomach, adipose, pancreas	Maturation and migration of dendritic cells, cell differentiation
P2Y12R	3q21–25	ADP	**2MeSADP >2MeSATP>ADP >ATPγS >ATP >ADPβS**	active metabolite of clopidogrel >pCMPS >AR-C69931MX >INS50589 >AZD6140 >2MeSAMP	Gαi	Platelets, brain, vascular smooth muscle	Platelet aggregation, thrombus growth and stabilization
P2Y13R	3q24–25	ADP	**2MeSADP >2MeSATP >ADP >ADPβS >ATPγS >ATP**	AR-C69931MX >Ap4A >Cangrelor (AR-C69931MX) >MRS2603 >Reactive Blue-2 >suramin	Gαi	Brain, spleen, bone marrow, platelets, epidemal cells, monocytes	Inhibition of ATP release from erythrocytes, regulation of hepatic high-density lipopotein endocytosis
P2Y14R	3q24–25	UDP-glucose	**UDP-glucose >UDP-galactose >UDP-glucuronic acid >UDP N-acethyl-glucosamine**	UDP	Gαi	Spleen and T- and B-lymphocytes, platelets, adipose tissue, intestine, skeletal muscle, spleen, lung, heart, peripheral blood mononuclear cells, pituitary, various brain regions	Chemotaxis, neuroimmune functions, dendritic cells activation regulation

1.4.1 P2Y1R

P2Y1R has been cloned from several species including humans, mice and rats (Ayyanathan *et al.*, 1996; Tokuyama *et al.*, 1995; Webb *et al.*, 1993). P2Y1R has a wide tissue expression profile including various brain areas and the peripheral nervous system, smooth muscle, endocrine tissue, adipocytes, kidneys, lungs and platelets (Burnstock *et al.*, 2004). P2Y1R stimulation is demonstrated with adenine nucleotides and its synthetic derivates. The natural agonist of P2Y1R is ADP, while conflicting data is reported about P2Y1R interaction with ATP. ATP is demonstrated to both stimulate the receptor as a partial agonist (Palmer *et al.*, 1998; Ralevic *et al.*, 1998) or to antagonize P2Y1R signal transduction (Hechler *et al.*, 1998; Leon *et al.*, 1997). This dual effect is thought to be caused by an ectoenzyme NTPDase2 that converts ATP to ADP on the cell surface (Alvarado-Castillo *et al.*, 2005). Two synthetic agonists 2-methylthio-ADP (2-MeSADP) and N-methanocarba-2-methylthio-ADP (MRS2365) are reported to have a potency on P2Y12R several times higher than ADP and are often used in research (Chhatriwala *et al.*, 2004; Waldo *et al.*, 2004). MRS2365 derivates MRS2179 and MRS2279 are used as potent antagonists of P2Y1R (Table 4) (Boyer *et al.*, 2002; Boyer *et al.*, 1998).

Currently, one of the most important physiological functions of P2Y1R is considered to be participation in platelet aggregation. Two purinergic receptors, P2Y1R and P2Y12R, are expressed on the platelet surface where they jointly facilitate blood coagulation. The first evidence of a P2Y1R role in blood clotting came with description of a prolonged bleeding phenotype in P2Y1R knockout mice (Leon *et al.*, 1999). Further investigation showed that ADP binding to P2Y1R leads to activation of PLCβ via the Gαq subunit of a G protein and PLCβ mobilizes intracellular calcium ions, changing platelet cell shape and insuring initiation of the coagulation process (Park *et al.*, 1999). A detailed scheme and description of P2Y1R and P2Y12R functions in platelet aggregation is in Figure 7.

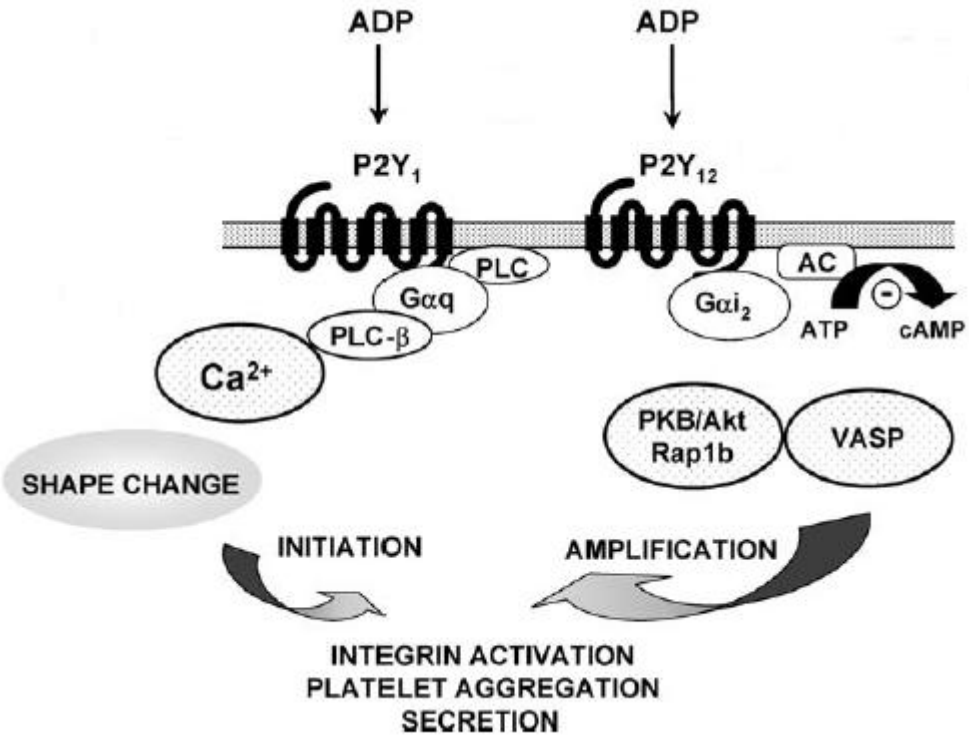

Figure 7. The platelet aggregation activation by P2Y1R and P2Y12R. After blood vessel injury, activated platelets bind to von Willebrand factor (Savage *et al.*, 1996) and release specific, primed granules upon recognition of collagen molecules in to the vessel wall. The secreted granules then discharge their contents of ADP, ATP and hormones into the blood circulation (Mills *et al.*, 1968). The liberated ADP binds to P2Y1R, which in turn induces activation of the G protein's Gαq subunit. Interaction of Gαq with PLCβ mobilizes intracellular calcium ions, changing platelet cell shape and initiating aggregation (Park *et al.*, 1999). Meanwhile, binding of ADP to P2Y12R prompts the Gαi subunit to reduce the intracellular concentration of cyclic adenosine monophosphate (cAMP). The subsequent increase in vasodilator-stimulated phosphoprotein and other enzymes ultimately leads to αIIbβ3 integrin receptor activation (Halbrugge *et al.*, 1990). (Figure and figure legend adapted from (Gachet, 2006)).

Much attention in the literature is given to the possible importance of P2Y1R in neuronal signalling. However, P2Y1R involvement in neuronal signalling is more complex than its function in platelet aggregation. Since P2Y1R is detected in many neuronal tissues a role in signal transduction is logical. Several reports have demonstrated a connection between Gαq activation and opening or closure of Ca^{2+} and K^{+} ion channels, the main pathways of neuronal signalling. For example, Gαq activation can lead to voltage-independent inhibition of Ca^{2+} flux via second messenger pathways (Filippov *et al.*, 2003). Implication of Gαq activation on K^{+} channels has also been observed (Huang *et al.*, 1998; Suh *et al.*, 2002). However, further studies are required to assess the practical application of these findings.

P2Y1R has been implicated in protection against development of atherosclerosis in studies of P2Y1R/apolipoprotein E double-knockouts, suggesting

involvement in prevention of *angina pectoris* and heart diseases (Hechler *et al.*, 2008).

P2Y1R is reported to form homooligomers when expressed in HEK293 cells. Agonist stimulation was demonstrated to strongly increases oligomer aggregation (Choi *et al.*, 2008). Several other studies investigated heterooligomerization between Gαi-coupled P2Y1R and Gαq-coupled adenosine 1 receptor (A1R). Heteromeric A1R/P2Y1R structures decrease in response to A1R agonists and antagonists, while increasing functional activation or inhibition by P2Y1R ligands. Experiments using PTX indicate that the Gαi subunit of a G protein is the primal signal transduction mechanism of the oligomer, but signalling via the Gαq subunit, usually by transducing A1R activation, is reduced (Nakata *et al.*, 2005; Yoshioka *et al.*, 2001).

Interesting facts of P2Y1R trafficking and oligomerization with P2Y11R were revealed by Ecke et al. The authors reported formation of P2Y1R/P2Y11R heteromers and sequential internalization of these complexes after activation by agonists. Intriguingly, P2Y11R is not able to internalize alone therefore, P2Y1R might be crucial factor for P2Y11R-mediated signalling (Ecke *et al.*, 2008).

1.4.2 P2Y12R

P2Y12R is reported in several species including humans, mice and rats (Foster *et al.*, 2001; Hollopeter *et al.*, 2001; Zhang *et al.*, 2001). The natural agonist of P2Y12R is ADP. Information about ATP action on P2Y12R is inconsistent. ATP is reported to stimulate the receptor with a lower affinity than ADP (Simon *et al.*, 2001; Zhang *et al.*, 2001) or to display antagonist properties (Kauffenstein *et al.*, 2004). The cause of this difference is not completely clear. Commonly used synthetic agonists of P2Y12R are 2MeSADP, which displays higher potency than ADP, and ADPβS, which has a lower potency than ADP (Abbracchio *et al.*, 2006). Several antagonists of P2Y12R, AR-C derivates and AZD6140 have also been developed (Ingall *et al.*, 1999; Storey *et al.*, 2007).

P2Y12R is expressed in various tissues including the central and peripheral nervous system, immune system, kidneys and platelets (Burnstock *et al.*, 2004). The main physiological function of P2Y12R as described in the literature is participation in blood coagulation. After binding of ADP to P2Y12R on the platelet cell surface, a conformational change in the receptor structure activates the Gαi subunit of a G

protein in the intracellular space. Gαi triggers inhibition of adenylyl cyclase and subsequently cAMP concentration in the cell decreases. This reduction leads to increased activity of several proteins such as vasodilator-stimulated phosphoprotein (VASP), which switches on expression of αIIbβ3 integrin receptor. The αIIbβ3 integrin receptor further binds fibrinogen, resulting in formation of a blood clot (Figure 7) (Eckly *et al.*, 2001; Halbrugge *et al.*, 1990; Kauffenstein *et al.*, 2001; Savage *et al.*, 1996).

P2Y12R signalling has been implicated in crosstalk with P2Y1R second messenger pathways. P2Y12R stimulation not only triggers Gαi adenylyl cyclase inhibition, but also increases Ca^{2+} ion concentration, thereby assisting P2Y1R-induced second messenger pathways (Hardy *et al.*, 2004).

Similar to P2Y1R, the P2Y12R is considered to be involved in regulation of neuronal signalling. In particular, the liberation of the Gβ/γ dimer that occurs after Gαi activation, which allows Gβ/γ to interact with various pathways leading to Ca^{2+} or K^{+} channel activation (Simon *et al.*, 2002). However, the role of these interactions is not generally clear and further investigations are needed.

Participation of P2Y12R in blood coagulation is considered a more important function because it is targeted by several clinically used antiplatelet drugs. Ticlopidine (trade name: Ticlid) and clopidogrel (trade name: Plavix) are two therapeutics that target P2Y12R and are frequently used for prophylactic purposes after coronary interventions like stenting or angioplastics to protect against thrombosis (Gurbel *et al.*, 1999; Jauhar *et al.*, 1999). Ticlopidine and clopidogrel belong to thienopyridine derivates. They bind to cystein residues in the extracellular loops, blocking agonist binding (Ding *et al.*, 2003). The drawback of these compounds is that enzymatic cleavage by hepatic P450 cytochromes in liver is required for creation of the active metabolite (Pereillo *et al.*, 2002; Savi *et al.*, 1992) and this process can delay drug effects. Up to 40% of patients treated with clopidogrel have resistance to the drug (Gurbel *et al.*, 2007) for various reasons such as genetic polymorphisms that affect drug absorbability (e.g. *ABCB1*) or enzymatic cleavage (*CYP3A4, CYP3A5, CYP2C19*), or physiological specificities that influence therapy effectiveness (Hulot *et al.*, 2006; Lau *et al.*, 2004; Simon *et al.*, 2009; Suh *et al.*, 2006). Because of these disadvantages, the search for next-generation P2Y12R antagonists is continuing. Novel compounds like cangrelor (compound code: ARC-69931 MCX), prasugrel

(compound code: CS-747) and elinogrel (compound code: PRT060128) are undergoing pharmacological evaluation (Ingall *et al.*, 1999; Sugidachi *et al.*, 2000; Ueno *et al.*, 2010).

P2Y12R receptors form homooligomers in human platelets. Intriguingly, upon stimulation by active state clopidogrel these oligomers disarrange into monomeric receptor forms. Furthermore, activation by the clopidogrel metabolite caused migration of P2Y12R out of lipid rafts (Savi *et al.*, 2006). This suggests that clopidogrel not only inhibits agonist binding to P2Y12R, but also might affect the effectiveness of signal transduction. As adenylyl cyclases are reported to be located in lipid rafts on the cell membrane (Ostrom *et al.*, 2001), removal of P2Y12R from its secondary messenger system could inhibit agonist signal transmission.

1.4.3 Other P2YRs

P2Y2R has a very wide tissue expression, and is detected in the nervous and immune systems, endocrine glands, gastrointestinal tract, bone tissues, skin and endothelial cells (Moore *et al.*, 2001). P2Y2R natural agonists are ATP and UTP, which display equal potencies. The functions of P2Y2R have mostly been suggested to be related to ion-exchange regulation, first for Cl^- ions in epithelial cells (Cressman *et al.*, 1999), and also Ca^{2+}, K^+ and Na^+ ions (Matos *et al.*, 2005; Pochynyuk *et al.*, 2008; Ryu *et al.*, 2010). P2Y2R is reported to form heterooligomers with A1R, with aggregation leading to an increase in the Gαq signalling pathway stimulated by either P2Y2R or A1R agonists, indicating a shift to higher P2Y2R signal transduction in these oligomers (Suzuki *et al.*, 2006).

The natural agonist of human P2Y4R is UTP. P2Y4R is widely expressed in the brain, gastrointestinal tract, smooth muscles and lungs (Communi *et al.*, 1995; Jin *et al.*, 1998; Moore *et al.*, 2001). The phenotype of knockout mice revealed that, similar to P2Y2R, P2Y4R is involved in Cl- ion regulation in epithelial cells (Robaye *et al.*, 2003).

P2Y6R has the highest affinity for UDP, however, it also binds UTP, ADP and ATP with reduced affinity (Communi *et al.*, 1996). This receptor is expressed in the spleen, gastrointestinal tract, smooth muscle and immune cells (Chang *et al.*, 1995). The functions of P2Y6R are not clear, but it is suggested to be involved in ion-exchange regulation, cell differentiation and proliferation (Schafer *et al.*, 2003; Warny

et al., 2001). P2Y4R and P2Y6R oligomerization has studied, demonstrating that both P2Y4R and P2Y6R form homoaggregates as well as heteromers (D'Ambrosi *et al.*, 2007).

P2Y11R is expressed in the immune system, gastrointestinal tract, kidneys and endothelial cells (Communi *et al.*, 1997; Moore *et al.*, 2001). This receptor has no ortholog in mice, therefore, no knockout studies have been performed and the physiological functions of P2Y11R remains unclear. Some indications have implicated P2Y11R in the maturation of dendritic cells and granulocyte differentiation, suggesting involvement in the immune response (van der Weyden *et al.*, 2000; Wilkin *et al.*, 2001). P2Y11R is the only P2YR containing an intron. Interestingly, P2Y11R is reported to form oligomers with P2Y1R and this process is crucial for receptor trafficking (Ecke *et al.*, 2008).

P2Y13R functions are also not entirely clear. It is expressed in spleen, brain, bone marrow, monocytes, erythrocytes and leucocytes (Communi *et al.*, 2001; Wang *et al.*, 2004). The primary natural agonist of P2Y13R is ADP. The physiological functions of the P2Y13R are not yet known, but some studies have demonstrated involvement in endocrine function and nociceptive mechanisms (Amisten *et al.*, 2010; Malin *et al.*, 2010).

P2Y14R is the only P2YR that is stimulated by sugar-bound nucleotides UTP-glucose, and with lower affinity, UTP-galactose. This receptor is expressed in adipose tissue, gastrointestinal tract, lungs, bone marrow, spleen and distinct brain areas (Chambers *et al.*, 2000; Moore *et al.*, 2003). P2Y14R is suggested to be involved in neuroimmune functions (Skelton *et al.*, 2003), but these findings require further study.

1.4.4 Functional characteristics of P2Y12R

Although P2Y12R is the target for the important clinical drug clopidogrel, the functional structures of P2Y12R are poorly understood. The first evidence of residues involved in P2Y12R functional activity were from Cattaneo and colleagues, who found two SNPs in positions R256 (rs121917885) and R265 (rs121917886) associated with platelet coagulation dysfunction (Cattaneo *et al.*, 2003). So far, few published mutagenesis studies have explored the ligand binding and activation of P2Y12R. Hoffman and colleagues generated R256K, R256A, R256D, S101A, Y259D, K280A mutants and the double mutant H253A/R256A of P2Y12R, and

showed that positions R256, Y259, H253 and K280 influence P2Y12R functional activation with 2-MeSADP. The R256A receptor variant had significantly reduced functional effects with the antagonist reactive blue-2, but not with cangrelor (Hoffmann *et al.*, 2008). Detailed analysis demonstrated the importance of positions R256 and S101 in interaction with different antagonists (Hoffmann *et al.*, 2009). Mao et al. investigated mutants F104S, Y109S, F198P, H253S, R256T, R256Q, R265W, S288P and the double mutant R256Q/R265W of P2Y12R for their activity after ADP activation (Mao *et al.*, 2010). Their results indicated the importance of F104S and S288P in reduced agonist activation. Positions R256 and R265 showed no involvement in ADP activation separately, but the double mutant R256Q/R265W had significantly decreased receptor activity. However, the single mutations R256Q, R256T and R265W and the double R256Q/R265W mutant demonstrated increased antagonist AR-C69931MX-mediated signalling (Mao *et al.*, 2010). Another mutagenesis study of thiol reagent effects on P2Y12R revealed a disulphide bridge formed by residues C17 and C270 to be important in the interaction between the thiol agent p-chloromercuribenzene sulfonic acid (pCMBS) and P2Y12R (Ding *et al.*, 2003).

The only mutagenesis report in which P2Y12R was expressed in yeast concentrated on the identification of residues that ensure the ground state of the receptor by generating untargeted randomised libraries. For example, the authors described position L115 of P2Y12R as important for constitutive receptor signalling (Schoneberg *et al.*, 2007). Functionally important residues of the P2Y12R are in Figure 8. Other information on the functional characteristics of P2Y12R can be deduced based on similarities to other purinergic receptors.

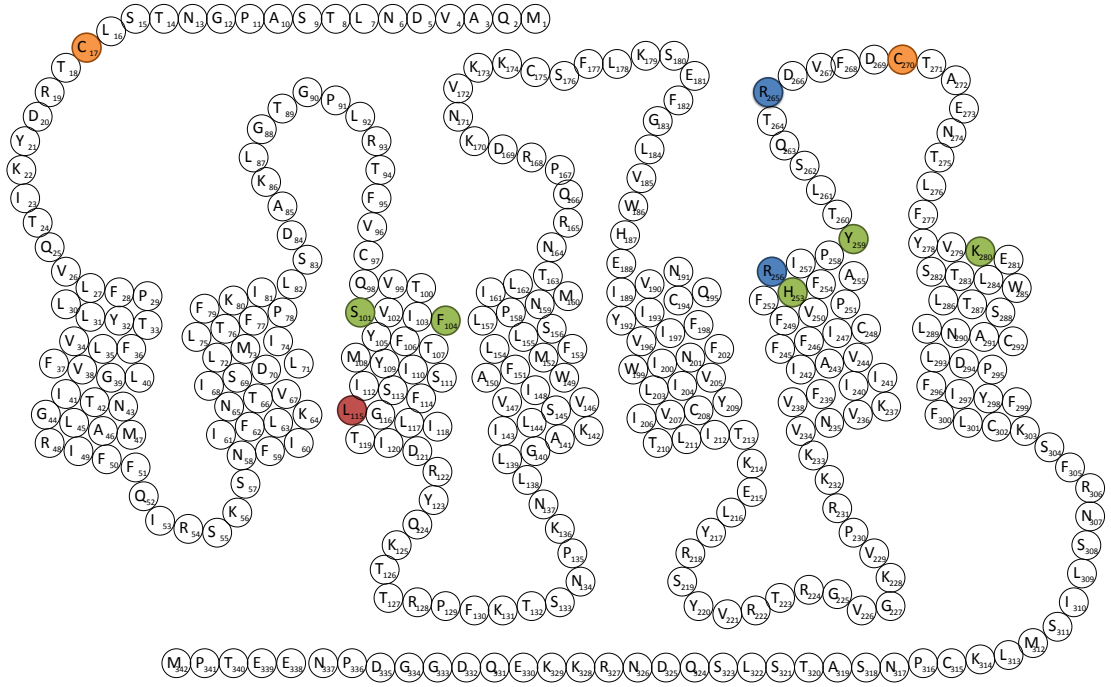

Figure 8. Schematic diagram of P2Y12R. Coloured amino acids are reported to be important using mutagenesis. Highlighted in blue are amino acids, that have been found in patients with blood coagulation disorder, in green - other residues responsible for receptor activation, in red – residue, that is responsible for constitutive activity of the receptor, in orange – cysteins, that interact with thiol groups of synthetic antagonists (Figure created by the author of the thesis, based on descriptions in literature).

The first complete three-dimensional homology model of P2Y12R was published in Protein Data Bank (PDB) and described in 2007 by Zhan et al. (Zhan *et al.*, 2007). This model was created using bovine rhodopsin (PDB entry 1HZX) as a template, but docking studies on the generated P2Y12R (PDB entry 1VZ1) failed to demonstrate ligand binding to the receptor in the possible binding pocket indicated by functional mutagenesis studies of P2Y12R and other P2YRs (Hoffmann *et al.*, 2008; Mao *et al.*, 2010; Moro *et al.*, 1998; Zylberg *et al.*, 2007). Previous homology modelling and docking of P2Y12R (using bovine rhodopsin PDB entry 1F88 as a template) were more consistent with ligand-receptor interaction data from the literature, but this model was not published in PDB (Costanzi *et al.*, 2004). A recent publication showed homology modelling of several P2YRs including P2Y12R using human β2 adrenergic receptor template (PDB entry 3SN6). These models had some correspondence to functional studies of P2YRs; for example, they indicated positively charged amino acid residues in the upper parts of TM domains of P2YRs. However,

the authors did not explore the ligand binding properties of P2Y12R in detail (Bhatnagar *et al.*, 2011).

1.4.5 Functional characteristics of other P2YRs

Functional properties of other P2YR receptors have been studied in more detail than P2Y12R. These data on other P2YRs can serve as a useful guide for description of P2Y12R.

Extensive P2Y1R mutagenesis studies have been carried out over the last decade. Residues from TM3, TM6, TM7 and EL2 are shown to be responsible for ADP-binding pocket formation. Positions F226, K280, Q307 and to lesser extent H132, Y136 and T222 of P2Y1R are important for receptor activation by the antagonist MRS2179 (Moro *et al.*, 1998). In experiments using the agonists 2-MeSATP and 2-MeSADP on P2Y1R mutants, neither ligand functionally activated R128A, R310A, or S314A and they showed decreased activity with the K280A and Q307A P2Y1R variants. Reduced P2Y1R activity was also seen in F131A, H132A, Y136A, F226A, or H277A replacement variants, but T221A and T222A substitutions in the receptor significantly lowered response to 2-MeSATP (Jiang *et al.*, 1997).

Hoffmann and colleagues studied the extracellular loops of P2Y1R and found that like many other GPCRs, P2Y1R has a disulphide bridge formed by residues C124 and C202 that is required for stabilizing the receptor during trafficking to the cell membrane. Two other disulphide bridge-forming cysteins C42 and C296 are crucial for 2-MeSADP-induced receptor activation. Mutagenesis of several charged amino acids in EL showed that most of them have small effect on receptor's functional activity (positions K125, R195, K196, K198, D204, R212, R285, D289, D300, R301). Only E209A and R287E mutants in EL2 and EL3 showed 1000-fold lower activity (Hoffmann *et al.*, 1999).

The role of the E209 position of P2Y1R has also been explored by homology modelling. An involvement in ligand attraction to the binding pocket by formation of possible meta-binding sites was proposed (Moro *et al.*, 1999).

The regions involved in functional activity and ligand binding in other P2YRs have been less well studied. P2Y11R mutagenesis demonstrated R106A, Y261A and R307A replacement variants are functionally inactive after ATP stimulation, R268A has significant reduction of potency, while F109I, E186A and R268Q show slight

reductions (Zylberg *et al.*, 2007). Hillman et al. reported that R272 and S296 are essential amino acids required for response to both ATP and UTP activation of P2Y2R (Hillmann *et al.*, 2009).

These studies have led to the overall characterization of the nucleotide binding pocket of P2YRs. The nucleotide base is believed to bind deep in the receptor cavity formed by TM3 and TM7, where this binding is promoted by Ser, Tyr, Phe and His residues, while phosphate groups are fixed in the upper parts of the TM6 and TM7 helices by Arg, Lys and Gln amino acids (Hillmann *et al.*, 2009; Ivanov *et al.*, 2006; Moro *et al.*, 1998; Zylberg *et al.*, 2007).

1.4.6 Genetic association studies of *P2RY12*

Many studies have attempted to establish P2YR functions by investigating a possible correlation between genetic variation at the candidate gene locus and distinct phenotypic traits. P2Y12R is the most extensively assessed P2YR in genetic association studies. The reason for this is the hypothesis that specific genetic variations of *P2RY12* could influence responsiveness of the receptor for clopidogrel therapy, and therefore selected SNPs might serve as markers for pharmacogenetic evaluation (Angiolillo *et al.*, 2005; Bura *et al.*, 2006; Fontana *et al.*, 2003b).

The first relevant information on the association of *P2RY12* genetic variants with clopidogrel effects was by Fontana et al. Who showed four SNPs of *P2RY12* in strong linkage disequilibrium: iC139T (rs10935838), iT744C (rs2046934), ins801A (rs5853517) and G52T (rs6809699). These were reported to be associated with decreased response to clopidogrel in healthy subjects. A tetrad of minor alleles of these SNPs was referred as haplotye 2 (H2) (Fontana *et al.*, 2003a). In the literature, genotyping of any of four of these polymorphisms is named H2, because of the strong LD between them. These results were replicated in another healthy volunteer group where the H2 homozygous haplotype was reported to have a slightly decreased response to clopidogrel (Bura *et al.*, 2006). The assumption of correlation between H2 and P2Y12R function was strengthened after a case-control study demonstrated association of H2 with peripheral artery disease (Fontana *et al.*, 2003b). Nonetheless, two other studies showed no association of H2 with platelet aggregation in patients after coronary stenting undergoing clopidogrel therapy (Angiolillo *et al.*, 2005; von Beckerath *et al.*, 2005). On opposing report showed that the H2 homozygous

genotype is associated with clopidogrel resistance after stenting (Staritz *et al.*, 2009). No association between the H2 and clopidogrel response was found in patients with coronary artery disease (CAD) (Bierend *et al.*, 2008), however, one study indicated association of CAD and platelet aggregation after clopidogrel treatment in a non-smokers group (Cavallari *et al.*, 2007). H2 was not associated with increased risk for cardiovascular events in either CAD (Schettert *et al.*, 2006) or acute myocardial infarction patients treated with clopidogrel (Simon *et al.*, 2009). Another report stated an association of H2 with deep venous thrombosis and pulmonary embolism, but no association with myocardial infarction or stroke (Zee *et al.*, 2008). The possible impact of H2 on occurrence of neurological events in patients treated with clopidogrel was also assessed in a study by Ziegler et al., that found no association with H2; however, another SNP 34C-T (rs6785930) in the *P2RY12* locus demonstrated association with ischemic cerebrovascular events (Ziegler *et al.*, 2005). In non-ST-elevation acute coronary syndrome patients, no correlation between H2 genotype and clopidogrel induced aggregation occurred (Cuisset *et al.*, 2007). Two studies on large patient groups were carried out by Rudez et al. These studies focussed not on the H2 haplotype, but tagged several haplotypes to completely cover the *P2RY12* locus. One study demonstrated that in patients undergoing clopidogrel treatment after percutaneous coronary intervention, the haplotype containing the minor alleles of rs10935842 and rs6787801 and the haplotype containing the minor alleles of rs10935842, rs6787801 and rs2044935 had an increased risk of target vessel restinosis (Rudez *et al.*, 2008). In the other study by Rudez et al., the haplotype containing the minor allele of rs6787801 was associated with decreased aggregation in response to clopidogrel in patients with CAD (Rudez *et al.*, 2009).

In spite of these studies, the significance of *P2RY12* variation in platelet aggregation remains unclear. Interestingly, Malek et al. postulated that the possible role of P2Y12R in responsiveness to clopidogrel might be limited not to variation in *P2RY12* alone but might include coexistence with a variation in *CYP2C19* responsible for metabolization of clopidogrel. Together, these genetic variations could have a significant effect on clopidogrel resistance and explain the controversies in the literature, if these possible effects were considered (Malek *et al.*, 2008). Another gene associated with a higher rate of cardiovascular events among patients treated with clopidogrel is *ABCB1*, which is responsible for its absorption (Simon *et al.*, 2009).

This indicates that research on *P2RY12* without considering the context of other genes related to clopidogrel pharmacodynamics does not lead to interpretable results (Malek *et al.*, 2008). A LD plot of the *P2RY12* region is in Figure 9.

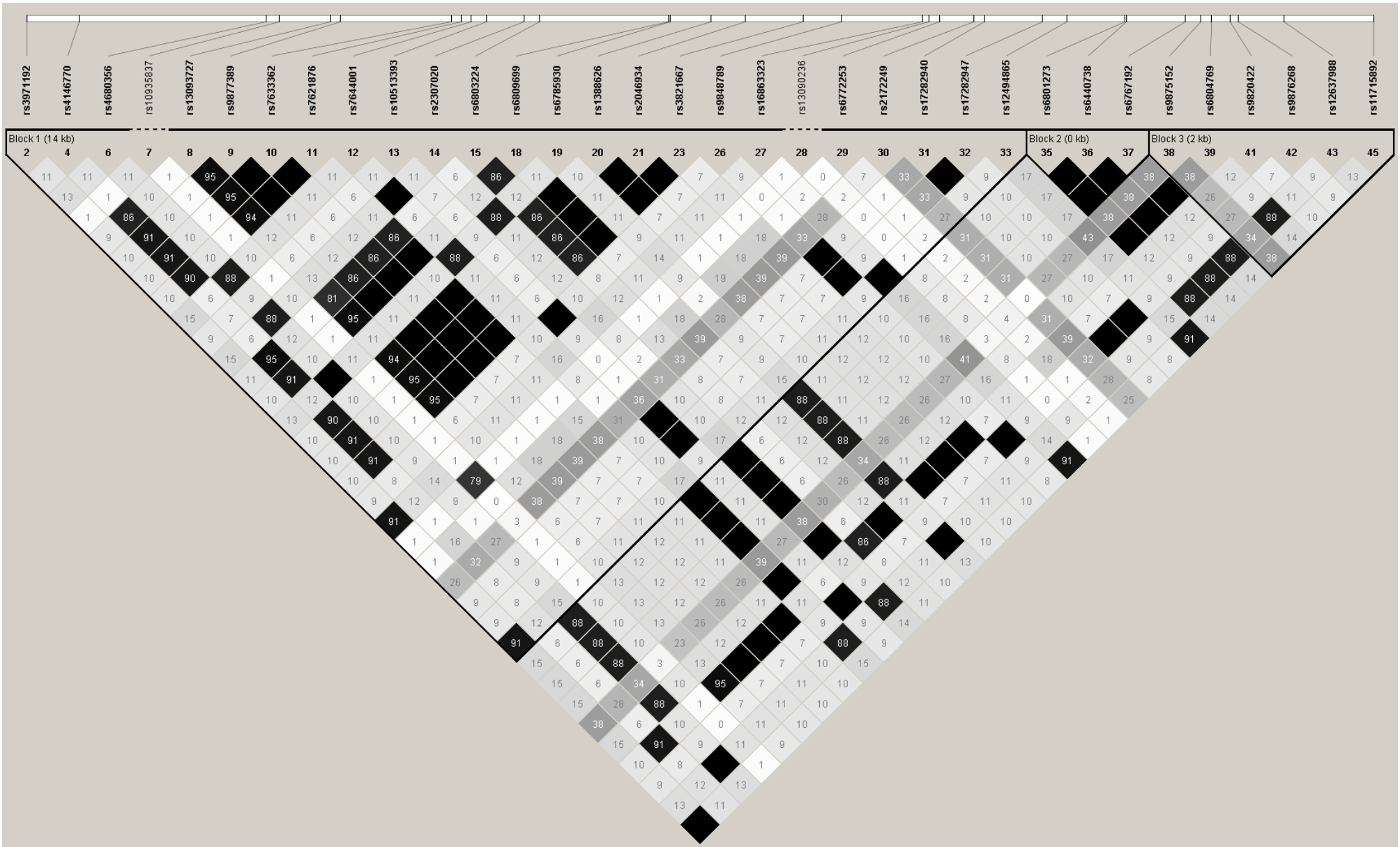

Figure 9. LD plot of the *P2RY12* region, created by the author of the thesis in HaploView 4.2 using HapMap project genotype frequency data (Rel#24; www.hapmap.org), 20 Kbp captured. The pairwise r^2 values are presented in each box. Black – strong evidence of LD, dark grey – moderate evidence of LD, light grey – low evidence of LD, white – uninformative.

1.4.7 Genetic association studies of *P2RY1*

P2Y1R has been suggested to correlate with coronary dysfunctions and discussed as possible target for antiplatelet therapies, since, with P2Y12R, it contributes to platelet aggregation (Gachet, 2006). So far, however, no antagonists are known to act via P2Y1R as used in clinical practice. Nonetheless, several studies explored the role of SNP variation at the *P2RY1* locus in relation to different phenotypes. rs701265 is located in coding region of *P2RY1* and is associated with higher platelet reactivity to ADP in healthy adults (Hetherington *et al.*, 2005). In contrast, another study on a similar but a half smaller study population detected no association of this polymorphism with platelet aggregation (Fontana *et al.*, 2005). This SNP showed no association with clopidogrel (Lev *et al.*, 2007; Sibbing *et al.*, 2006) or aspirin (Lev *et al.*, 2007; Li *et al.*, 2007) resistance. However, one study revealed a significant association of rs701265 with platelet responsiveness to aspirin in myocardial infarction (MI) patients after percutaneous coronary intervention (Jefferson *et al.*, 2005). A second frequently genotyped SNP of *P2RY1* is rs1065776, which is located in the upstream non-coding region of the *P2RY1*. This polymorphism is reported to significantly correlate with aspirin resistance in Caucasian and Chinese populations (Jefferson *et al.*, 2005; Kunicki *et al.*, 2009a; Li *et al.*, 2007). In a healthy adult study group, rs1065776 had significant effects on platelet response to collagen binding and ADP (Kunicki *et al.*, 2009b). Extensive exploration by genotyping of seven SNPs (rs2579133, rs17451266, rs9289876, rs1065776, rs701265, rs16864613, rs12497578) in the *P2RY1* locus and correlation of the genotypes to administration of ticagreol (a clopidogrel analog) on platelet reactivity showed no significant association between these polymorphisms and the studied phenotype (Storey *et al.*, 2009). Therefore, the function of *P2RY1* genetic association in relation to phenotypic traits remains unclear. A LD plot of the *P2RY1* region is in Figure 10.

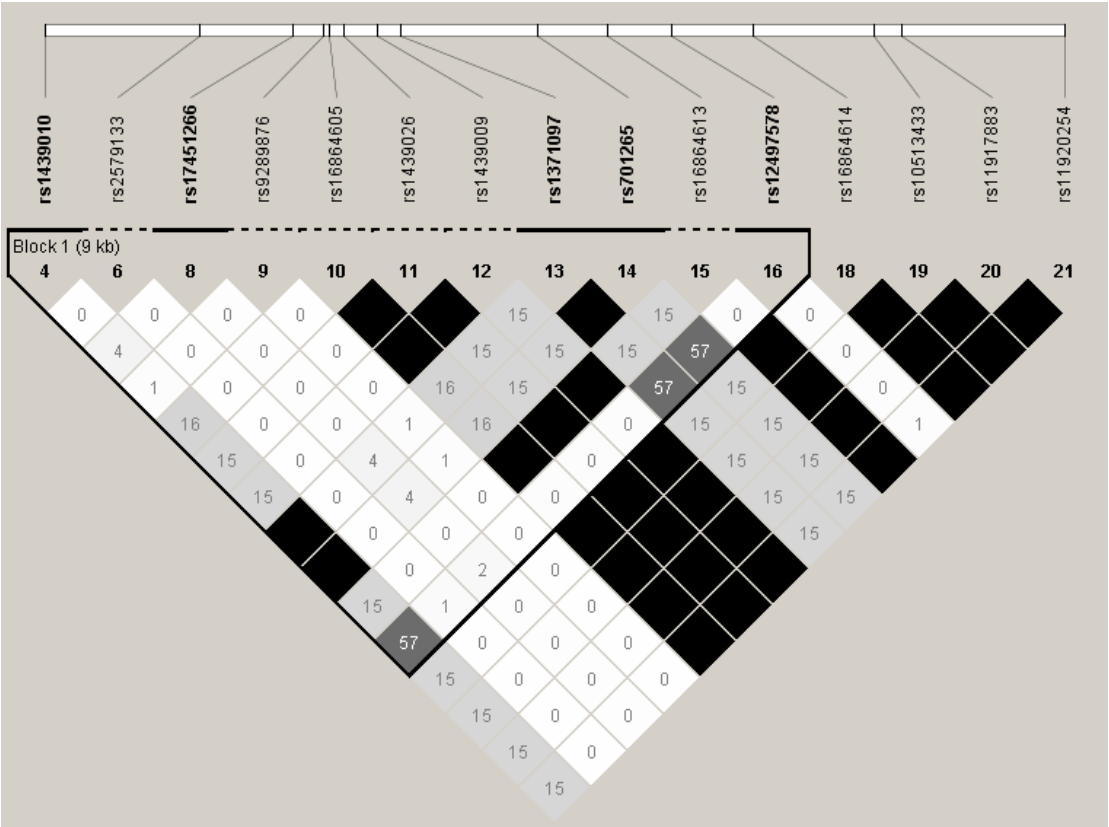

Figure 10. LD plot of the *P2RY1* region, created by the author of the thesis in HaploView 4.2 using HapMap project genotype frequency data (Rel#24; www.hapmap.org), 20 Kbp captured. The pairwise r^2 values are presented in each box. Black – strong evidence of LD, dark grey – moderate evidence of LD, light grey – low evidence of LD, white – uninformative.

1.4.8 Genetic association studies of other *P2RYs*

The other *P2RYs* have not been as thoroughly studied by genetic association as *P2RY1* or *P2RY12*, but reports have linked polymorphisms in *P2RYs* with phenotypic features. For example, the genetic variant R312S (rs3741156) of *P2RY2* is more common in cystic fibrosis patients than in healthy controls. Furthermore, functional studies have shown that this variation has an effect on intracellular Ca^{2+} release that might influence the cystic fibrosis phenotype (Buscher *et al.*, 2006). The role of P2Y2R in development of atherosclerosis and thus association of *P2RY2* polymorphisms with myocardial and cerebral infarction has been explored in a Japanese population, which revealed that the specific haplotypes CA (rs4382936 and rs10898909) and TCA (rs1783596 and rs4382936, rs10898909) are associated with myocardial infarction in Japanese men. Another genotype (rs1783596-rs4382936-rs10898909) correlates with the incidence of cerebral infarction in Japanese women (Wang *et al.*, 2009b; Wang *et al.*, 2009c).

One report explored the association of the A87T polymorphism (rs3745601) in *P2RY11* in relation to acute myocardial infarction. This SNP is significantly associated with MI and C-reactive protein levels indicating the possible effects of P2Y11R in inflammation processes that contribute to coronary dysfunctions (Amisten *et al.*, 2007). Amisten and colleagues examined the Met-Thr-156 (no rs code) polymorphism in *P2RY13* association with myocardial infarction, but found no significant association (Amisten *et al.*, 2008).

2 MATERIALS AND METHODS

2.1 *Escherichia coli* strain

DH5α - *F–, phi80dlacZDelta M15, Delta(lacZYA–argF), U169, deoR, recA1, hadR17(rk–, mk+), gal–, phoA, supE44, Lambda–, thi-1, gyrA96m relA1*

Purchased from Invitrogen, USA.

2.2 *Saccharomyces cerevisiae* strains

MMY23 - *MATa his3 leu2 trp1 ura3 can1 gpa1Δ::Gi far1Δ::ura3 sst2Δ::ura3 Fus1::FUS1-HIS3 LEU::FUS1-lacZ ste2Δ::G418^R*

MMY28 - *MATa his3 leu2 trp1 ura3 can1 gpa1Δ::Gs far1Δ::ura3 sst2Δ::ura3 Fus1::FUS1-HIS3 LEU::FUS1-lacZ ste2Δ::G418^R*

Both were kindly provided by Dr. S.J. Dowell (Olesnicky *et al.*, 1999).

2.3 Mammalian expression cell lines

HEK 293 (EBNA) - Human embryonic kidney cell line (Invitrogen, USA)
BHK – Baby hamster kidney cell line (Invitrogen, USA)

2.4 Primers

Primers for minisequencing were purchased from Biotez (Germany), all other primers from Metabion (Martinsried, Germany). Primer sequences are presented in Table 5, Table 6 and Table 7.

Table 5. Primers used for mutagenesis of *MC4R* and *P2RY12*

Primer name	**Primers for *MC4R* randomisation**
MC4R D126rand Fw	CATTGATAATGTCATTNNNTCGGTGATCTGTAGC
MC4R D126rand Rs	GCTACAGATCACCGANNNAATGACATTATCAATG
Primer name	**Primers for *P2RY12* randomisation**
P2RY12R E181rand Fw	CATGCCAGACTAGACCGAANNNTGATTTAAGGAAAGAGCA
P2RY12R E181rand Rs	TGCTCTTTCCTTAAATCANNNTTCGGTCTAGTCTGGCATG
P2RY12R R256rand Fw	TCAGGGTGTAAGGAATNNNGGCAAAATGGAAAGGAAC
P2RY12R R256rand Rs	GTTCCTTTCCATTTTGCCNNNATTCCTTACACCCTGA
P2RY12R R265rand Fw	CAGTGCAGTCAAAGACATCNNNGGTTTGGCTCAGGGTGT
P2RY12R R265rand Rs	ACACCCTGAGCCAAACCNNNGATGTCTTTGACTGCACTG
P2RY12R K280rand Fw	TAACCACAGAGTGCTCTCNNNCACATAGAACAGAGTATT
P2RY12R K280rand Rs	AATACTCTGTTCTATGTGNNNGAGAGCACTCTGTGGTTA
Primer name	**Primers for site-direct mutagenesis of the *MC4R***
MC4R V103I Fw	CTGGTGAGCGTTTCAAATGGATCAGAAACCATTATCATCACCCTATTAAACAGTACAGATACGG
MC4R V103I Rs	CCGTATCTGTACTGTTTAATAGGGTGATGATAATGGTTTCTGATCCATTTGAAACGCTCACCAG
MC4R S127L Fw	CAGTGAATATTGATAATGTCATTGACTTGGTGATCTGTAGCTCCTTGCTTGCATCC
MC4R S127L Rs	GGATGCAAGCAAGGAGCTACAGATCACCAAGTCAATGACATTATCAATATTCACTG
MC4R V166I Fw	CCAGTACCATAACATTATGACAGTTAAGCGGATTGGGATCATCATAAGTTGTATCTGGGCAGC
MC4R V166I Rs	GCTGCCCAGATACAACTTATGATGATCCCAATCCGCTTAACTGTCATAATGTTATGGTACTGG
MC4R I251L Fw	CCAATATGAAGGGAGCGATTACCTTGACCATCCTGCTTGGCGTCTTTGTTGTCTGCTGGGCC
MC4R I251L Rs	GGCCCAGCAGACAACAAAGACGCCAAGCAGGATGGTCAAGGTAATCGCTCCCTTCATATTGG

Table 6. Primers used for genotyping of *P2RY1*

rs code	**Primers for amplification**
rs2579133	Fw - ACG TTG GAT GCT TTA TCT CAT AAA TCA GGG TCT C Rs - ACG TTG GAT GAG AAT GGG AAA ATC ATT GG
rs17451266[a] rs9289876	Fw - ACG TTG GAT GAT TAA CAT GGA ATG CTT AGG G Rs - ACG TTG GAT GCT GAC TTC ACT CAG AAG TTT GTT
rs1439009	Fw - ACG TTG GAT GGT GCT AGA TTC CAT TCT TGT G Rs - ACG TTG GAT GAC GGT TAT AAT TCC AAA GTG AC
rs1065776	Fw - ACG TTG GAT GTC TAA GGT AGG GAG GAG GAA Rs - ACG TTG GAT GTT TGA ACG ACG AGG AGA C
rs701265	Fw - ACG TTG GAT GGC TTT GAT TTA CAA AGA TCT GG Rs - ACG TTG GAT GAC ATG GAA AGG GAT GTA AGA
rs12497578	Fw - ACG TTG GAT GGC CTA TGT AGA TTG AGG ATT TG Rs - ACG TTG GAT GAA TCA CTG CTT TGG AAG GT
rs code	**Primers for minisequencing**[b]
rs2579133	bioTCT TAC CTA (L)CA GCT CCC AAC TTC
rs17451266	bio TT ATT CCC ACA AGG C(L)A AAT TTT
rs9289876	bioGA AAT GTA CAC TT(L) GTC CAG ATA CCA
rs1439009	bioA GAA AAC GAG TTT GTC (L)GT AAA TGT
rs1065776	bioCCA GGA C(L)A ACC CGG ACC
rs701265	bioC AGC AAA AAC (L)GT CAG TAC AAT GAT
rs12497578	bioT CTT CAT TGA TCT TAG (L)GG CCA

[a] for both SNPs the same primers were used.
[b] bio - indicate biotin cap; (L) - photolinker cleavage site.

Table 7. Primers used for sequencing of *MC4R* and *P2RY12*

Primer name	**Primers for *P2RY12* amplification and sequencing**
P2RY12Fw	CCAAAAGCTTATGCAAGCCGTCGACAACCT
P2RY12Rs	TACTCGAGTTACATTGGAGTCTCTTCATTT
Primer name	**Primers for *MC4R* amplification**
MC4R pcr Fw1	GGAGGAAATAACTGAGACG
MC4R pcr Fw2	TATGCTGGTGAGCGTTTC
MC4R pcr Rs1	CCAATCAGGATGGTCAAG
MC4R pcr Rs2	TTCAGGTAGGGTAAGAGT
Primer name	**Primers for *MC4R* sequencing**
MC4R seq Fw1	CTGAGACGACTCCCTGAC
MC4R seq Fw2	GGATCAGAAACCATTGTC
MC4R seq Rs1	CCAATCAGGATGGTCAAG
MC4R seq Rs2	TGTTCCTATATTGCGTGC

2.5 Vectors

For yeast experiments yeast shuttle vector p426TEF (American Type Culture Collection, USA) was used. For expression and activation experiments of MC4R in mammalian cells vector pcDNA3 (Invitrogen, USA) was used. For confocal microscopy of mammalian cells vector pCEP4-GFP-C was used, this vector was obtained by modification of pCEP4 (Invitrogen, USA) with insertion of enhanced green fluorescent protein (EGFP) gene at the 3'end of the multiple cloning site.

2.6 PCR-directed mutagenesis

Mutant libraries of *MC4R* and *P2RY12* and site-direct mutant variants of *MC4R* were obtained by mutagenesis PCR using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, Canada) using mutant primers shown in Table 5 following manufacturers' protocol. Heterogeneity of the randomised products was confirmed by sequencing (described below).

2.7 DNA agarose gel electrophoresis

DNA agrose gel electrophoresis was performed in TAE buffer (40 mM Tris–acetate pH 8.3, 20 mM sodium acetate, 1 mM EDTA) on 1.2 % agarose (Fermentas, #R0491) gels containing ethidium bromide (1 μg/ml).

2.8 Restriction of DNA

The restriction of DNA was carried out in reaction mix that contained: 1 μl of 10X R restriction buffer (Fermentas, #BR5), 5–10 μl of DNA (volume depended on the type and concentration of the DNA), 0.5 μl of each of the required restriction enzymes (HindIII (Fermentas, #ER0501) and/or XhoI (Fermentas, #ER0691)), and sterile distilled water up to 20 μl. The reaction mix was incubated for 3 h at 37°C.

2.9 Ligation of DNA fragments

The ligation of DNA fragments was carried out in reaction mix that contained: 1 μl of 10× ligation buffer, 1 μl of 10 mM rATP, 1 μl of vector DNA, 8 μl of fragment DNA, 1 μl of T4 DNA ligase (Fermentas, #EL0011). The reaction mix was incubated for 3 h at 22°C.

2.10 DNA extraction and purification from agarose gel

DNA gel extraction and purification was performed with the Silica Bead DNA Purification and Gel Extraction Kit (Fermentas #K0513) following the manufacturers protocol.

2.11 Preparation of transformation competent *E. coli* cells

E.coli cells from a frozen stock of DH5α strain were inoculated into 6 ml of LB media (10 g/l Bacto tryptone, 5 g/l Bacto yeast extract both from Difco Laboratories, USA and 10 g/l sodium chloride from Sigma- Aldrich Co, USA) containing no antibiotics and were incubated overnight at 37ºC with aeration (~200 rpm). Then, the overnight culture (~1 ml) was inoculated into 150 ml of Psi media (20 g/l Bacto tryptone, 5 g/l Bacto yeast extract, 5 g/l magnesium sulphate, at pH 7.6, adjusted using potassium hydroxide) and the culture was then incubated at 37ºC with aeration (~200 rpm) until the OD_{590} = 0.3–0.4. Then culture was incubated on ice for 15 min and pelleted for 5 min at ~5000 rmp. 20 ml of TfbI (30 mM potassium acetate, 100 mM rubidium chloride, 10 mM calcium chloride, 50 mM magnesium chloride, 15% v/v glycerol, at pH 5.8, adjusted using acetic acid, all purchased from Sigma-Aldrich, Germany) was added to cell pellet and cells were resuspended, incubated on ice for 15 min and pelleted for 5 min at ~5000 rmp. The supernatant was discarded and 2 ml of TfbII (10 mM MOPS, 75 mM calcium chloride, 10 mM rubidium chloride, 15% glycerol, at pH 6.5, adjusted using sodium hydroxide, all purchased from Sigma-Aldrich, Germany) were added to the pellet and resuspended. Finally, cells were incubated on ice for 15 min, snap-frozen in liquid nitrogen in 100 μl aliquots and stored at –70ºC.

2.12 Transformation of *E. coli* cells

An eppendorf containing transformation competent E. coli cells was defrozen on ice. 10 μl of ligation mix or 1 μl of plasmid was added to the cells and incubated on ice for 30 min. The heat shock was carried out for 45 s at 42°C and then cells were incubated

on ice for 2 min. The cells were transferred to 0.9 ml of pre-warmed LB (10 g/l Bacto tryptone, 5 g/l Bacto yeast extract both from Difco Laboratories, USA and 10 g/l sodium chloride from Sigma- Aldrich Co, USA) broth containing no antibiotics, incubated for 1 h at 37ºC with aeration (~200 rpm) and pelleted for 3 min at ~3000 rpm. Most of the supernatant was carefully discarded leaving ~100 µl of media. The cells were then resuspended and inoculated on LB agar (10 g/l Bacto tryptone, 5 g/l Bacto yeast extract both from Difco Laboratories, USA and 10 g/l sodium chloride, 15 g/l Bacto agar both from Sigma- Aldrich Co, USA) plates containing ampicillin (Sigma-Aldrich, Germany) (1 mg/ml).

2.13 Plasmid DNA extraction

E.coli cells from a single colony or a frozen stock was inoculated into 6 ml (Miniprep) or 150 ml (Midiprep) of 2×YT media (16 g/l Bacto tryptone, 10 g/l Bacto yeast extract both from Difco Laboratories, USA and 5 g/l sodium chloride from Sigma-Aldrich Co, USA) containing ampicillin (Sigma-Aldrich, Germany) (1 mg/ml) and was incubated overnight at 37ºC with aeration (~200 rpm). For Miniprep DNA extraction the GeneJet™ Plasmid Miniprep Kit (Fermentas #K0503) was used and for Midiprep DNA extraction the JETSTAR 2.0 Plasmid Midiprep Kit (Genomed #210050) was used. Plasmid DNA extraction was carried out following manufacturers' protocol.

2.14 Sequencing of mutant *P2RY12* and *MC4R* vector constructions

Isolated mutant vector constructions were sequenced using primers P2RY12Fw and P2RY12Rs for *P2RY12* and MC4R seq Fw1, MC4R seq Fw2, MC4R seq Rs1, MC4R seq Rs2 for *MC4R* (Table 7). Sequencing mix contained - 5 µl Millipore H_2O, 2 µl sequencing buffer, 0.5 µl BigDye (both from Applied Biosystems, USA), 0.5 µl primer using the following conditions denaturation at 95^0C for 5 min, 25 cycles of 95^0C for 15 sec, 50^0C for 5 sec, 60^0C for 4 min. Products were precipitated by addition of 30 µl of 96% ethanol and 1 µl of 7.5 M ammonium acetate (Sigma-Aldrich, Germany) and incubated at room temperature for 10 min and then centrifuged at ~14000 rmp for 15 min, supernatant was removed and 150 µl of 70% ethanol added and centrifuged for 5 min at ~14000 rmp. After centrifugation supenatant was removed and precipitant air-dried. Sequencing analysis was performed on 3130xl Genetic Analyzer (Applied Biosystems, USA). Analysis of the sequences was done by ContigExpress software as implicated in VectorNTI (Invitrogen, USA) and manually verified.

2.15 Yeast maintanence and transformation

Yeast cells from frozen stock of MMY23 or MMY28 were grown overnight in 4 ml YPD medium (20 g/l bactotriptone, 10 g/l yeast extract, both from Difco Laboratories, USA) at 30°C with aeration (~200 rpm). The transformation in yeasts with desired vector was carried out in 100 mM lithium acetate (Sigma, Germany) and 0.2 mg/ml salmon sperm DNA (Sigma, USA), yeasts were heat shocked for 40 min and then inoculated on SC medium agar plates containing 1.92 g/l yeast synthetic drop-out medium supplement without uracil, 1.7 g/l yeast nitrogen base, 5 g/l ammonium sulfate, 2 % glucose, 16.67 g/l agar (all purchased from Sigma, Germany). The agar plates were grown for 24 – 48 h at 30°C. The colonies from selective agar plates were inoculated in 2 ml liquid SC medium (recipe above) lacking uracil and grown

overnight, culture were stored frozen in 25 % glycerol (Sigma-Aldrich, Germany) at -70°C.

2.16 Receptor functional activation in yeast system

A single colony from selective agar plates or culture from frozen stock was inoculated in 2 ml liquid SC medium (recipe above) lacking uracil and grown overnight at 30°C with aeration (~200 rpm). Functional activation was carried out in 96-well plates (Starsted, USA) in 200 µl SC medium (recipe above) also containing 2 mM AT (3-Amino–1,2,4-triazole from Sigma, Germany) that is a competitive inhibitor of imidazoleglycerol-phosphate dehydratase for control of background synthesis of histidine, 100 µl/ml 10xBU salts (70 g/l $Na_2HPO_4 \bullet 7H_2O$, 30 g/l NaH_2PO_4 ph7 adjusted by 2 M NaOH, all purchased from Reahim, Russia), 0.1 mg/ml chlorophenol red-β-D-galactopyranoside (Roche, Germany). For functional activation of MC4R – αMSH, ACTH (both from PolyPeptide group, France), THIQ (kindly provided by the Institute of Organic Synthesis of Latvia) were used, for P2Y12R – ADP, 2MeSADP (both from Sigma, Germany), AR-C 66096 (Tocris Bioscience, UK) – were used. Lignads were applied in a concentration interval from 30 µM to 10 nM depending on experiment. Cells were diluted with activation medium to concentration of 250 cells/µl. Additionally, negative control for each clone was performed in activation medium without ligand to determine background activity of the receptor variants. Cells were grown in room temperature for 24h or longer, depending from experiment. OD at 595 nm was measured on Victor$^3{}_{TM}$V reader (PerkinElmer, USA). Data analysis was performed in GraphPad Prism (GraphPad Software, USA). Data sets were normalized according to highest and lowest values, and transformed using function X=log(X), and EC_{50} values and SEM were calculated automatically. Significance of difference between wt receptor EC_{50} and mutant receptor EC_{50} values was estimated by *t*-test.

2.17 Transfection and cultivation of mammalian expression cell lines

Prior to transfection BHK or HEK 293 cells were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (both purchased from Sigma, Germany) to 70% monolayer density at 37^0C. 1 µl of transfection vector DNA was diluted in 100 µl of DMEM and 2 µl of TurboFect^{TM} *in-vitro* Transfection Reagent (ThermoScientific, USA). The transfection mix was incubated for 15 min in room temperature and then added to cell monolayer and grown for 24 h for microscopy experiments and for 36 h for cAMP response assay.

2.18 cAMP response assay

After transfection cells were grown for 36 h and transferred to 384-well plate in final concentration 1 x 10^4 cells per well. The expressed receptors were functionally activated with serially diluted concentrations of αMSH, ACTH (both from PolyPeptide group, France) and AGRP (83-132) (Phoenix Pharmaceuticals, USA). Intracellular cAMP concentration was measured using LANCE cAMP 384 kit (PerkinElmer, USA) following the manufacturer's protocol and measurements were done on plate reader VictorV3 (PerkinElmer, USA). The cAMP concentrations were quantified by comparison to a standard curve of the control concentrations of cAMP provided in a kit. Data analysis was performed in GraphPad Prism (GraphPad

Software, USA) as described above (Chapter 2.16 Receptor functional activation in yeast system).

2.19 Preparation of confocal microscopy samples

BHK cells 24 h after tansfection grown in 12-well plate containing microscopy slips were labelled with AlexaFluor 633-labeled wheat germ agglutinin to visualize cell membrane (Invitrogen, USA) for 2 min washed with 1×PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and fixed with formaldehyde (Sigma, Germany) for 10 min at 37^0C and washed again with PBS. After that cell nucleus was visualized with DAPI 6 µl of 1.5 µg/ml (Sigma-Aldrich, Germany). The microscopy slips were placed on microscopy slide and cover slip were placed on the cell droplet and inspected with confocal fluorescent microscope Leica DM 600B (Leica, Germany). Each receptor mutant was microscoped at least after two independent transfections.

2.20 QSAR

The QSAR (quantitative structure–activity relationship) was performed using by SIMCA P-11 software (Umetrics AB, Sweden). EC_{50} values from activation experiments were converted to logarithmic scale and inactive receptor variants were assigned with dramatically decreased receptor activity EC_{50} value 10^5 nM. Descriptors by Sandberg et al. (Sandberg *et al.*, 1998) and Gottfries et al.(Gottfries *et al.*, 2009) were used, charge was also added as descriptor (1 – positively; -1 – negatively; or 0 – not charged). Descriptors represented - hydrophobibity/hydrophilicity (z1), steric/bulk properties (z2), polarity (z3) and electronic effects (z4 and z5) of amino acids, and t-Rig – rigidity, t-Flx – flexibility (Gottfries *et al.*, 2009; Sandberg *et al.*, 1998). Different combinations of the descriptors were used to obtain a model that best correlated amino acid properties with receptor activity of the studied mutant libraries. Models were generated by orthogonal partial least square regression (OPLS) and characterized by calculation of activity variation (R^2) and predictive ability (Q^2) calculated in SIMCA P-11.

2.21 Homology modeling and docking

Due to the highest sequence homology 2.6 Å resolution X-ray structure of human adenosine 2a receptor (A2aR) (PDB entry 3EML) (Jaakola *et al.*, 2008) was used as a template for P2Y12R modeling. First, the P2Y12R and A2aR sequences were aligned using GPCR AlignmentBuilder available at www.gpcr.org, this web-tool implements a special alignment algorithm for class A (rhodopsin-like) GPCRs, that is designed taking into consideration specific characteristics of the transmembrane regions of rhodopsin-like GPCRs as described by (Mirzadegan *et al.*, 2003). Then obtained sequence alignment was manually adjusted in MolSoft ICM-Pro 3.5, and 3D model of P2Y12R created by ICM-Homology modeling algorithm. This algorithm incorporates - alignment of P2Y12R sequence to template structure and replacement of non-identical sequence residues simultaneous global energy optimization is done and the prediction of side chain torsion angles is carried out (based on biased probability Monte Carlo (BPMC) conformation alignment and optimization (Abagyan *et al.*, 1994; Totrov *et al.*, 1997)). The quality of the generated model is verified by specialized ICM function that predicts possible deviation of backbone between the model and the template (Cardozo *et al.*, 2000). The obtained 3D homology model of

P2Y12R was used for ADP docking by ICM-Docking algorithm implemented in MolSoft ICM-Pro 3.5. Briefly, the location of binding pocket was identified and receptor energy maps generated with grid cell size 0.5 Å. After that flexible ligand docking was done using multiple conformations of the ADP and different locations within designated binding pocket. As the last step the refinement of the developed model was done for all docked conformations of the ADP to adjust receptor conformation to the ligand. The docking model of 3D P2Y12R with the lowest free energy value was considered the most corresponding to binding *in-vivo*.

2.22 Study group for *P2RY1* genotyping

The STrengthening the REporting of Genetic Associations (STREGA) guidelines (Little *et al.*, 2009) were followed to plan study design and statistical analysis. Groups of cases and controls were selected from the Latvian Genome Data Base (LGDB) national biobank of health and genetic information collected for adult residents of Latvia (over 18 years old). Health status of the participants was designated by certified physicians using International Classification of Diseases (ICD)-10 codes. Anthropometric data such as weight and stature were obtained by direct measurement, demographic information (ethnic, social, and environmental characteristics) and familial health status had been obtained by questionnaire-based interview. Written confirmed consent had been acquired from all LGDB participants. The study protocol was approved by the Central Medical Ethics Committee of Latvia.

Case-control groups were selected from all LGDB participants (n=11342) recruited from 2003 to 2009. From the initial pool of subjects all individuals with cancer (ICD: from C00 to C97), renal conditions (ICD10: from N00 to N99) or rare diseases were excluded, to avoid overwhelming confounding influences on the analysis. Also all individuals with missing important phenotypic data were excluded. The selection of MI cohort was based on several criteria – MI diagnoses were verified by professional cardiologists according to symptomology at presentation, electrocardiogram results and biomarkers in blood. A total of 1781 individuals comprised the final MI study group (females, n=536 and males, n=1245). For analysis, 263 females and 592 males were randomly selected from the MI group. The healthy group of controls were selected from the initial pool of LGDB participants excluding all individuals with chronic diseases and heart and coronary disease conditions (ICD10: from I20 to I70), leaving 1725 healthy adults from this 855 group controls were randomly selected maintaining the same sex proportion as in the case group.

2.23 Genotyping with MALDI-TOF

DNA sample stocks from LGDB (description above - chapter 2.22 Study group for *P2RY1* genotyping) were diluted into 96-well PCR plates to final concentration 28ng per well using the Freedom Evo robotic workstation (Tecan, Männedorf, Switzerland). PCR primers for amplification of genomic DNA are presented in Table 6. Amplification mix contained - 1 mM DB buffer, 2.5 mM $MgCl_2$, 0.5 units (U) HotFire polymerase, 0.2 mM dNTP mix (all from SolisBioDyne, Tartu, Estonia) and 0.3mM each primer. PCR was done using following conditions - denaturation at 5 min at 95^0C, 40 cycles of amplification 30 sec at 95^0C, 30 sec at 55^0C and 1 min at 72^0C and final extension at 72^0C for 5min. PCR products were visualized on agarose gel electrophoresis. After that degradation of unused dNTP was done with shrimp alkaline phosphatase (Fermentas, Lithuania) following the producer's protocol.

Minisequencing primers were designed using the CalcDalton program (Kirsten *et al.*, 2006), the primers contained a biotin cap on 5' –end and a photolinker cleavage site, all primer sequences are presented in Table 6. The minisequencing mix contained - 0.5 mM 10xC buffer, 1.25 mM $MgCl_2$, 1 U TermiPol DNA polymerase (all from SolisBioDyne), 0,2 mM ddNTPs (Fermentas, Lithuania), 1 mM each primer, and 5 µl of the purified PCR product. Minisequencing conditions were - denaturation of 2 min at 95^0C, followed by 99 cycles for 10 sec at 95^0C, 10 sec at 55^0C, 10 sec at 72^0C, and final extension at 72^0C for 5min. Minisequencing products were precipitated on streptavidin-coated 384-well plates (Biotez, Germany) and subjected to UV exposure for 15 min. 1µl of the precipitated DNA minisequencing product was diluted in 1 µl of 3–hydroxypicolinic acid matrix (70 mM and 40 mM diammonium hydrogen citrate) and spotted on an MTP AnchorChip 400/384TF (Bruker Daltonik, Germany) and finally air-dried at room temperature. Matrix-assisted laser desorption/ionization (MALDI)-TOF MS analysis was carried out on a Autoflex MS (Bruker Daltonik, Germany) using a maximum of 40 nitrogen laser shots with frequency of 16.7 Hz for each sample spot. Mass calibration was performed with the low molecular weight oligonucleotide calibration standard (1000-4000 Da; Bruker Daltonik, Germany). For genotyping of rs2579133 and rs701265: 4000–5000 Da was used, for rs1439009, rs1065776 and rs12497578: 1300–3600 Da was used and for rs17451266 and rs9289876: 1900–4200 Da was used. Genotyping data was analysed with the Genotools SNP Manager software (Bruker Daltonik, Germany) and manually verified.

2.24 Study group for *MC4R* and *FTO* genotyping

The STrengthening the REporting of Genetic Associations (STREGA) guidelines (Little *et al.*, 2009) were followed to plan study design and statistical analysis. Case and control groups were selected from LGDB (for more detailed description of LGDB see above chapter 2.22 Study group for *P2RY1* genotyping). For case and control groups subjects were selected from LGDB participants recruited from 2003 till May of 2011 (n=16503). The participants with missing important phenotypic data, with body mass associated conditions like cancer (ICD: from C00 to C97), hyperthyriosis related diseases (ICD: from E00 to C07), and other (ICD: from E20 to E27) and professional athletes were excluded. Than stratification according to BMI was carried out - normal weight (BMI 18.5 – 24.9) (n=2129) and obese (BMI > 30) (n=4096). For obesity study group 380 subjects having the highest BMI in LGDB were selected. For control group we randomly selected 380 individuals from normal weight group, maintaining the same sex proportion and average age as in the case group.

2.25 Genotyping of the *MC4R* and *FTO* with RT-PCR

DNA sample stocks from LGDB (description above - chapter 2.22 Study group for *P2Y1R* genotyping) were diluted into 96-well PCR plates to final concentration 28 ng per well using the Freedom Evo robotic workstation (Tecan, Männedorf, Switzerland). Genotyping was carried out with an Applied Biosystems TaqMan SNP Genotyping Assay (Applied Biosystems, USA). PCR mix contained 4.75µl TaqMan Genotyping Mix (Applied Biosystems, USA), 5µl Millipore H2O (Millipore) and 0.25 µl of SNP genotyping assay for rs17782313 ID:C_32667060_10, for rs11642015 ID:C_2031268_20, for rs9939609 ID:C_30090620_10 and for rs62048402 custom made probe was used (labelled on 5' end with VIC and FAM) (Applied Biosystems,

USA). The reaction was performed on a ViiA^{TM} 7 Real-Time PCR System (Applied Biosystems, USA) using cycling conditions - initialization at 60^0C for 1min and 95^0C for 10 min, 40 cycles of 92^0C for 15 sec and 60^0C for 1 min, final step at 60^0C for 1min. AutoCaller 1.1 (Applied Biosystems, USA) software was used to designate genotypes, samples with unsuccessful genotyping were removed.

2.26 Sequencing of *MC4R*

For the sequencing of complete coding region of the *MC4R* first amplification of the interested region by two primer pairs of the MC4RpcrFw1 and MC4RpcrRs1, MC4RpcrFw2 and MC4RpcrRs2 (Table 7) was carried out by standard polymerase chain reaction, mix contained 1 mM DB buffer, 2.5 mM $MgCl_2$, 0.5 units (U) HotFire polymerase, 0.2 mM dNTP mix (all from SolisBioDyne, Tartu, Estonia), 0.3 mM each primer, and 28 ng template DNA. PCR conditions were denaturation at 95^0C for 5 min, 40 cycles of amplification at 95^0C for 30 sec, 55^0C for 30 sec, and 72^0C for 1 min, and final extension at 72^0C for 5 min, reaction was carried on a Veriti96 ThermalCycler (Applied Biosystems, USA). PCR products were visualized on agarose gel electrophoresis and degradation of unused dNTP and primers was carried out using shrimp alkaline phosphatase and exonuclease I (Fermentas, Lithuania) according to the manufacturer's protocol. For sequencing primers MC4RseqFw1 and MC4RseqRs1, MC4RseqFw2 and MC4RseqRs2 were used (Table 7). Sequencing PCR mix contained 5 µl Millipore H_2O, 2 µl sequencing buffer, 0.5 µl BigDye (both from Applied Biosystems, USA), 0.5 µl primer using the following conditions denaturation at 95^0C for 5 min, 25 cycles of 95^0C for 15 sec, 50^0C for 5 sec, 60^0C for 4 min. The purification of PCR products was done by Sefadex following the manufacturer's protocol (Sigma, Germany). Sequencing analysis was performed on 3130xl Genetic Analyzer (Applied Biosystems, USA). Analysis of the sequences was done by ContigExpress software as implicated in VectorNTI (Invitrogen, USA) and manually verified.

2.27 Statistical analysis of genotyping data

Statistical analysis of genotyping data was performed with Plink 1.06 software (Purcell *et al.*, 2007) (http://pngu.mgh.harvard.edu/purcell/plink/). The possible deviation from HardyWeinberg equilibrium was estimated as implemented in Plink 1.06. To compare characteristics between case and control groups the chi-square and Student's *t*-test were used. The possible association between distinct phenotype and genotype was estimated by either logistic or linear regression, dependent on studied parameter. In case of the *P2RY1* association was adjusted for the cofactors of sex, age, BMI, smoking, alcohol consumption and physical activity, but in case of the *MC4R* and *FTO* association was adjusted for sex and age. For *P2RY1* also haplotype association and frequencies of haplotypes were calculated in Plink 1.06. LD plot was generated using the HaploView software 4.2. (Barrett *et al.*, 2005). Power calculations were performed using Quanto v.1.2.3 (Gauderman *et al.*, 2006) (http://hydra.usc.edu/gxe/). The allele frequencies estimated in our study were applied. For *P2RY1* population risk for MI was set at 0.01, but for *MC4R* and *FTO* population risk of obesity was set at 0.2 and α was set at 0.05. An additive mode of inheritance was assumed for power calculations.

2.28 Statistical analysis of microscopy images

Statistical analysis to determine cell surface expression of mutant MC4R variants confocal microscopy images were performed as previously described (Fridmanis *et al.*, 2010). Cells transfected with the desired receptor construct were separately examined for presence of EGFP fluorescence and cell membrane labelling with AF-WGA. Two particular transfections were done for each MC4R receptor construct and three distinct cells from each transfection were analysed. 18 cross section images were taken for the studied cell. The presence of the receptor variant in the cell membrane was determined by detection of EGFP/AF-WGA fluorescence intensity ratio at multiple - randomly selected points in the surface membrane. 20 points were picked for a single cell and their EGFP/AF-WGA fluorescence intensity ratios were quantified using Leica Confocal Software (LCS-Lite v2.61). The uniformity of the obtained intensity ratios was inspected with Kruskal-Wallis test (α set at 0.05) as implemented in GraphPad Prism software (GraphPad software, USA), if the significant variation was detected in the data set, outlayers were determined by Dunn's multiple comparison test (α set at 0.05) (GraphPad software, USA). In cases when significant differences were detected with more than two data sets, these data were replaced with new data from repeated transfection. Median values of fluorescence intensity of different receptor variants were compared using Kruskal-Wallis test (α set at 0.05).

3 RESULTS

3.1 Expression of Human Melanocortin 4 Receptor in *Saccharomyces cerevisiae*

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Expression of Human Melanocortin 4 Receptor in *Saccharomyces cerevisiae*

Research Article

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Abstract: The melanocortin 4 receptor (MC4R) is involved in the regulation of energy homeostasis and is known as one of the major hypothalamic regulators of food intake. Several studies have shown that replacement of aspartic acid at position 126 of the MC4R abolishes the ligand binding. We used the modified yeast *Saccharomyces cerevisiae* strain MMY28 to functionally express the MC4R and characterise the importance of this amino acid for ligand based activation of the receptor. The efficiency of the functional expression system was estimated by activation with αMSH, ACTH and THIQ and compared with cAMP response in mammalian cells. We generated the library of MC4R mutants randomised at the amino acid position 126. Recombinant MC4R clones were screened for the αMSH induced activity in yeast. From 9 different amino acids obtained only the natural aspartic acid displayed the ligand dependent activity of MC4R. The MC4R variants with glutamic acid and leucine at position 126, however, displayed higher background activity than other amino acid substitutions. The results suggest that the yeast expression system is suitable for screening of the MC4R receptor ligands and that the substitution of aspartic acid at position 126 of MC4R by different amino acids functionally inactivates the receptor.

Keywords: *G-protein coupled receptors • Saccharomyces cerevisiae • Melanocortin receptors • Functional screens*

Abbreviations

ACTH - adrenocorticotropin hormone;
AT - 3-Amino–1,2,4-triazole;
GPCR - G protein-coupled receptors;
MC4R - melanocortin 4 receptor;
MSH - melanocyte-stimulating-hormone;
THIQ - tetrahydroisoquinoline.

1. Introduction

The melanocortin type 4 receptor (MC4R) is a member of the G- protein coupled receptor family. Five different melanocortin receptors have been discovered in humans [1-4]. Natural agonists of MCRs are specific peptides – melanocortins that are enzymatically cleaved from a single polypeptide precursor – proopiomelanocortin [5-7]. Different melanocortins, α-melanocyte-stimulating-hormone (αMSH), βMSH, adrenocorticotropic hormone (ACTH) and γMSH act as agonists on MC4R [3,4,8-11].

The MC4R is mostly expressed in the ventromedial, dorsomedial, paraventricular and arcuate hypothalamic nuclei regions of the central nervous system, controlling food intake [10,12-14]. It has been shown that MC4R knock-out mice have severe adiposity that is caused by a hyperphagic phenotype [15]. To a lesser extent, the MC4R is involved in the regulation of different autonomic, neuroendocrine, anti inflammatory, stress and sexual functions [12,16,17].

Since the main role of the MC4R in an organism is the control of feeding behaviour and energy homeostasis it has become a potential drug target for adiposity treatment. A number of novel agonists and antagonists activating the MC4R have been described, and different studies conducted to characterize MC4R receptor-ligand interactions [18-23].

So far the functional studies of the MC4R have revealed two conformationally important binding sites of the melanocortin pharmacophore His-Phe-Arg-Trp. The first binding site is the negatively charged conformation pocket of MC4R, formed by E100, D122

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and D126 [18,24], which is interacting with the amino group of arginine residue from the pharmacophore sequence. The second binding site is the hydrophobic cavity that is formed by F184, F202, F261, F262 and F284 residues of the MC4R [25] (Figure 1). It has been shown that phenylalanine and tryptophan residues of the pharmacophore sequence are involved in strong hydrophobic interaction with MC4R in this pocket [23].

Previously the importance of D126 residue in the MC4R has been shown by functional characterization of mutant human MC4R containing the substitutions with alanine and asparagine at this position, and mutant mouse MC4R containing the substitution of the corresponding amino-acid with lysine. All substitutions abolished the ligand binding properties of the receptor [18,25].

Expression of GPCRs in yeast *Saccharomyces cerevisiae,* which is engineered to couple with the mammalian GPCRs and requires activation of the receptor for growth, has been used to pharmacologically characterize a number of various receptors [26-31]. The advantages of such an approach in GPCR research include low cost propagation, automation of the screening and a low level of false positive results that are common in mammalian cells due to endogenous expression of ligands or related receptors. Another advantage of the growth dependant yeast screening system is the possibility to select the variants of the functional receptor from the randomized expression libraries [32,33]. So far functional expression of the melanocortin receptors in yeasts has not been described.

In this study we demonstrate the functional expression of MC4R in yeasts and perform the characterization of the library of MC4R constructs with randomized amino acid at position 126.

2. Experimental Procedures

2.1 Mammalian cell culture and cAMP assays

HEK 293 cells were grown in Dulbecco's Modified Eagle's medium (Sigma, USA). Human MC4R cloned in pcDNA3 vector was transfected using TurboFect™ *in vitro* transfection reagent (Fermentas, Lithuania) following the producer's protocol. The transfected HEK 293 cells were diluted and distributed in 384-well plates to concentration 1 x 10^4 cells/well. The cells were incubated with the serially diluted concentrations of αMSH and ACTH. Intracellular cAMP levels were measured using LANCE^{tm} cAMP 384 Kit (PerkinElmer, USA) following the recommendations of the manufacturer on Victor$^3{}_{TM}$V reader (PerkinElmer, USA). The cAMP concentrations were quantified by comparison to a standard curve of the known amounts of cAMP provided in the kit.

2.2 Cloning and construction of mutagenised MC4R library

A human MC4R gene was cloned from human genomic DNA using MC4R-Fw (ATTGGGAAGCTTATGGTGAACTCCACCCACCGT) and MC4R-Rs (AAGCTCGAGTTAATATCTGCTAGACAAGTCA)

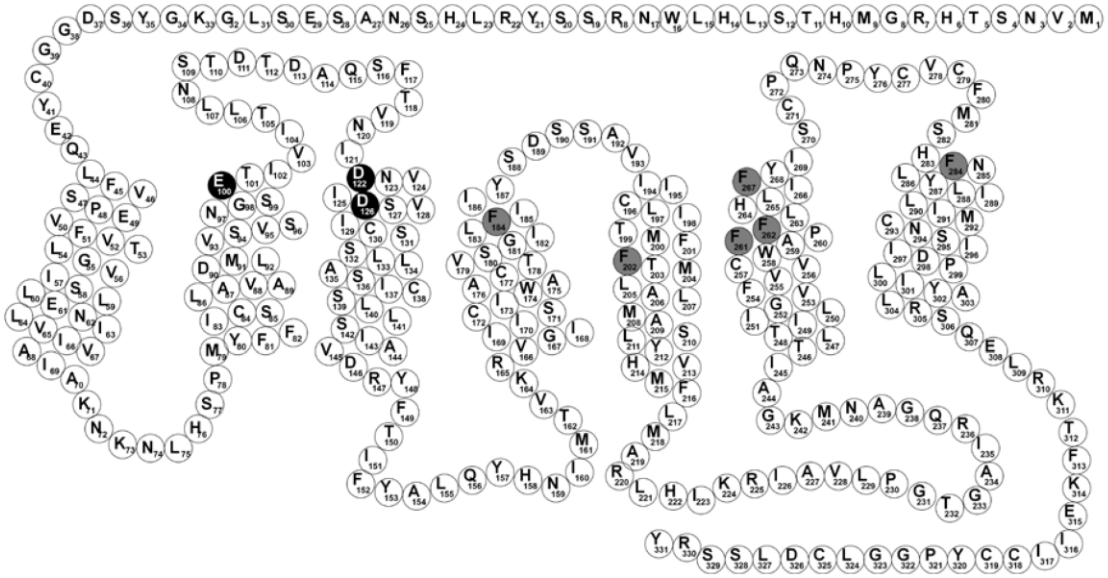

Figure 1. Residues of MC4R involved in receptor – melanocortin interaction. Residues shaded in black are amino-acids involved in formation of the acidic ligand binding pocket. Those shaded in grey are amino-acids involved in formation of the hydrophobic ligand binding pocket.

primers which contained *Hind*III and *Xho*I restriction sites respectively (underlined).

The mutagenised library of MC4R was constructed by PCR using *Pfu* polymerase (Fermentas, Lithuania) and oligonucleotides D126rand-Fw (CATTGATAATGTCATTNNNTCGGTGATCTGTAGC) and D126rand-Rs (GCTACAGATCACCGANNNAATGACATTATCAATG) (Operon, Germany) containing randomized trinucleotide (NNN) that corresponds to amino acid D126 of MC4R. The mutagenised PCR product was directly sequenced using primers MC4R-Fw and MC4R-Rs to confirm heterogeneity of randomization using 3130xl Genetic Analyser (Applied Biosystems, USA). The randomized library of the MC4R was cloned in *HindIII/XhoI* sites of the yeast shuttle vector p426-TEF that contains the URA gene for auxotrophic selection [34].

2.3 Yeast transformation

Yeast *Saccharomyces cerevisiae* strain MMY28 (MATa his3 leu2 trp1 ura3 can1 gpa1Δ: Gs far1Δ::ura3 sst2Δ::ura3 Fus1::FUS1-HIS3 LEU::FUS1-lacZ ste2Δ::G418^R) was kindly provided by Dr S.J. Dowell. The yeast strain expresses the chimeric yeast Gα/human G_sα subunit, and has a receptor activation system fused with a specific signalling pathway to reporter genes enabling β-galactosidase production and histidine synthesis.

Yeast cells were grown overnight in 4 ml YPD medium (20 g/l bactotryptone, 10g/l yeast extract, both purchased from Difco Laboratories, USA). The transformation of yeasts with a randomized library was carried out in 100 mM lithium acetate (Sigma, Germany) and 0.2 mg/ml salmon sperm DNA (Sigma, USA), yeasts were heat shocked for 40 min and then harvested and transferred to Petri plates containing SC medium agar supplemented with 1.92 g/l yeast synthetic drop-out medium without uracil, 1.7 g/l yeast nitrogen base, 5 g/l ammonium sulfate, 2% glucose, 16.67 g/l agar (all purchased from Sigma, Germany).

2.4 Functional activation of receptor variants

After transformation, colonies from selective agar plates were harvested in 2 ml of liquid SC medium lacking uracil and grown overnight. Functional activation was carried out in 96-well plates (Sarstedt, USA) in 200 ml SC medium without histidine and uracil, but containing 2 mM AT (3-Amino–1,2,4-triazole by Sigma, Germany) which is a competitive inhibitor of imidazoleglycerol-phosphate dehydratase used to suppress the background synthesis of histidine, 10xBU salts (70 g/l Na2HPO4.7H2O, 30 g/l NaH_2PO_4 adjust to pH 7 with 2 M NaOH, all purchased from Реахим, Russia), 0.1 mg/ml chlorophenol - red-β-D-galactopyranoside (Roche, Germany) and various concentrations of αMSH, ACTH (PolyPeptide group, France) and THIQ (kindly provided by the Institute of Organic Synthesis of Latvia). Cells were diluted within an activation medium to a concentration of 250 cells/ml. Negative control of each clone was used containing activation medium without ligand to determine the background activity of receptor variants. The concentration of αMSH, ACTH and THIQ varied from 30 mM to 10 nM. Cells were grown at room temperature for 24 h. OD at 595 nm was measured using a Victor$^3{}_{TM}$V reader (PerkinElmer, USA). All activation assays were performed in duplicate and were repeated at least 3 times.

2.5 Data analysis

Data analysis was performed using GraphPad Prism software (GraphPad Software, USA). Data sets were normalized according to the highest and lowest values, and transformed using the function X=log(X). EC_{50} values were calculated automatically.

3. Results

The human MC4R was expressed in modified *Saccharomyces cerevisiae* strain MMY28, which has a specific reporter system that upon activation of the expressed receptor allows the growth on medium lacking histidine. In addition, the β-galactosidase reporter gene permits the quantitative estimation of functional activity using the enzymatic colour reaction. In these yeast cells, agonist mediated MC4R activation induced the expression of the β-galactosidase gene in a dose-dependent manner. To validate the functional activity of MC4R and estimate the efficiency of the expression, yeast cells were activated by two endogenous ligands, αMSH, ACTH and a small molecule agonist THIQ (Figure 2a, Table 1).

	EC_{50}±SEM (nM)	
Ligand	*S. cerevisiae* [a]	HEK293 [b]
αMSH	269.0±28.0	5.81±1.9
ACTH	223.9±0.2	6.81±1.1
THIQ	490.4±11.8	nd

Table 1. Effects of αMSH, ACTH and THIQ on stimulation of wt MC4R activity.

nd – not detected
a- calculated from β-galactosidase production levels in Saccharomyces cerevisiae;
b- calculated from cAMP production levels in HEK293 cells

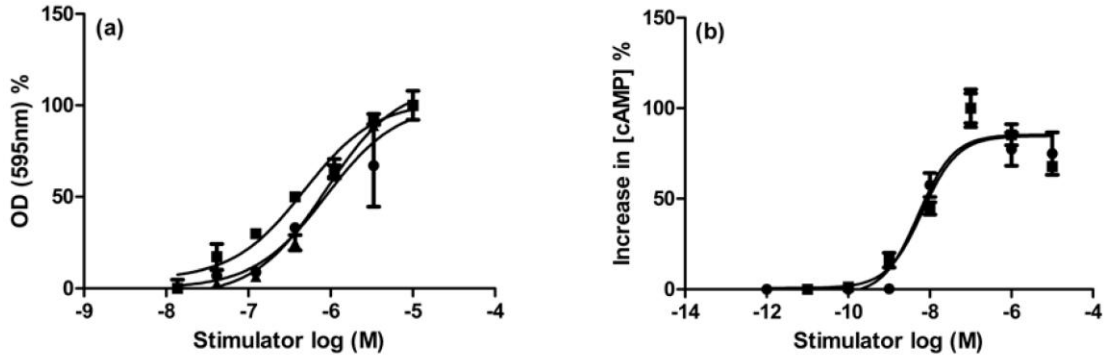

Figure 2. MC4R activation curves: (a) reporter gene expression in *Saccharomyces cerevisiae* strain MMY28 activated with αMSH (●), ACTH (■), THIQ (▲); (b) cAMP response curves in HEK293 cells activated with αMSH (●), ACTH (■). cAMP and OD are expressed as a percentage with the basal level set to 0% and the highest level in each data set to 100%.

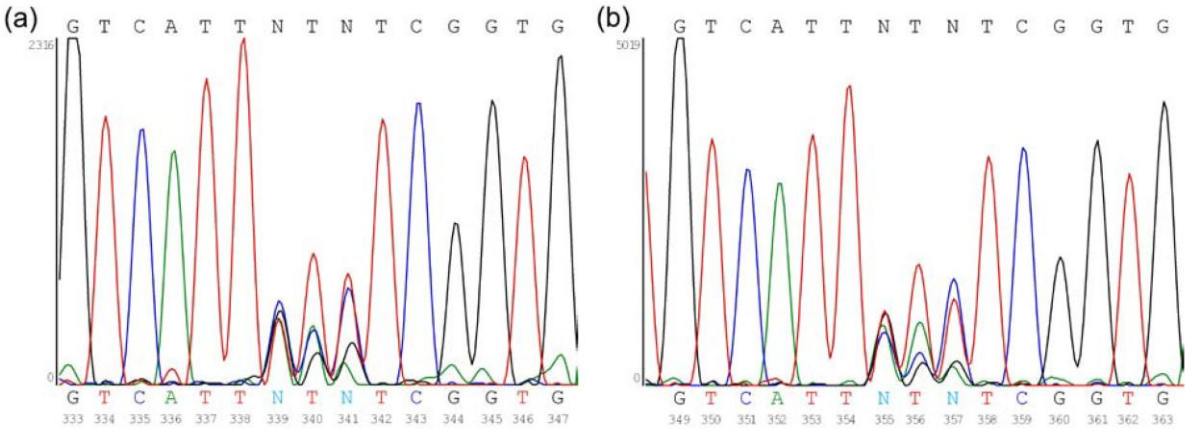

Figure 3. Sequence chromatograms representing the randomisation of MC4R at position 126: (a) sequence of PCR fragment; (b) sequence of the plasmid isolated from the pooled *E. coli* clones.

The ligand affinity curves and the obtained EC_{50} values show that in the yeast expression system the MC4R has a higher affinity for αMSH and ACTH as compared to THIQ. In order to compare the pharmacological properties of the MC4R expressed in the yeasts with those of mammalian cells, we performed a cAMP estimation in HEK293 cells transiently transfected with the MC4R upon incubation with αMSH and ACTH (Figure 2b). The basal level of cAMP was 0.34 pmol/10^4 cells while the maximal level of cAMP upon stimulation with the highest concentrations of αMSH and ACTH was 80 nmol/10^4 cells and 125 nmol/10^4 cells respectively.

The potency of MC4R to induce cAMP production upon activation in HEK293 cells was characterized by EC_{50} values that were similar to the previously published ones [35]. These values were 46 and 32 times lower than EC_{50} values of the αMSH and ACTH induced reporter gene expression in the yeast expression system respectively (Table 1), indicating that the yeast expression system has sufficient sensitivity for MC4R ligand testing.

The library of MC4R expression plasmids with various amino acid codons at position 126 was constructed by site directed mutagenesis using synthetic oligos with randomized nucleotides at specific positions. The heterogeneity of the obtained randomized library was confirmed by sequencing both: the PCR product before the cloning in yeast shuttle vector p426TEF and the plasmid isolated from the pooled *E. coli* cells after transformation (Figure 3).

Since we did not succeed in our initial attempt to perform functional selection of yeast clones containing the active receptor variants on ligand containing agar plates (data not shown), we continued with characterization of individual clones.

Fifty clones where selected and screened by direct sequencing. Twenty-four of all yeast clones analyzed contained more than one different plasmid as revealed from the presence of multiple sequencing chromatogram peaks at randomized positions. As many as 26 sequencing chromatograms contained no additional peaks at the corresponding positions, indicating the presence of plasmid with unique codon. In total, 14 different codons encoding 9 amino-acids were identified. Representation of these amino-acids in the pool of clones as well as the number of individual codons is shown in Table 2.

The obtained clones were further analysed by functional activation in the liquid yeast cultures supplemented with serial dilutions of αMSH and 2 mM AT for suppression of the background activation. All obtained wt MC4R clones were active upon stimulation and displayed similar EC_{50} values. At the same time none of the mutant MC4 receptors was able to induce the reporter gene expression (Figure 4, Table 3).

The same observation was made during the activation with THIQ (data not shown) indicating the importance of aspartic acid at position 126. Nevertheless, the glutamic acid and leucine variants displayed the increased levels of the basal reporter gene expression compared to other mutants. To confirm that this activity is not due to some physiological properties of the specific yeast cell clone or changes in plasmid sequence, we performed

Codon	Number of codons	Amino acid	Side chain characteristics
ATT	4	Asparagine	Polar, hydrophilic, neutral
GAC	5	Aspartic acid	Polar hydrophilic charged (-)
TGC	1	Cysteine	Polar, hydrophobic, neutral
GAA	1	Glutamic acid	Polar hydrophilic charged (-)
GGG	1	Glycine	Aliphatic, neutral
GGT	1	Glycine	
ATC	1	Isoleucine	Aliphatic, hydrophobic, neutral
ATA	1	Isoleucine	
CTA	2	Leucine	Aliphatic, hydrophobic, neutral
CTT	2	Leucine	
TTT	3	Phenylalanine	Aromatic hydrophobic neutral
TTC	1	Phenylalanine	
GTT	1	Valine	Aliphatic hydrophobic neutral
GTC	2	Valine	

Table 2. List of codons at amino-acid position 126 of MC4R identified from sequencing of individual clones.

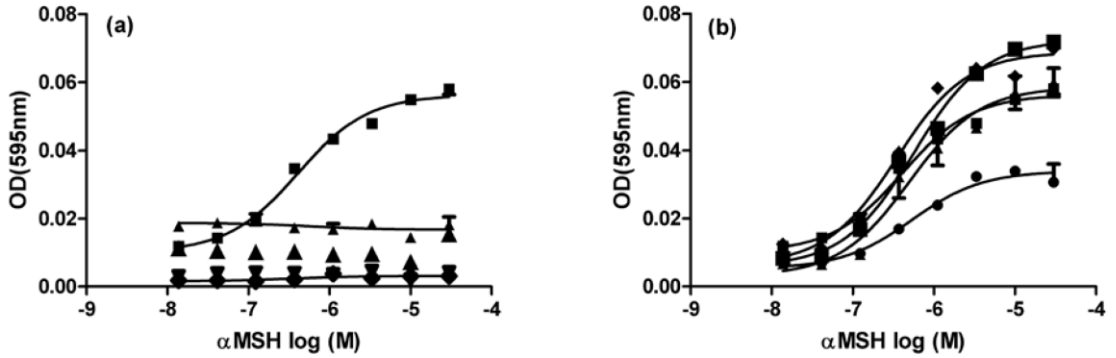

Figure 4. MC4R activation curves of individual MC4R codon variants: (a) wild-type (■) glutamic acid GAA (▲), leucine CTT (▲), phenylalanine TTT (▲) and phenylalanine TTC (♦); (b) several aspartic acid GAC (●), (▲), (▼), (■) variants.

the isolation of several recombinant MC4R constructs containing leucine and glutamic acid and retransformed them (as previously described by [27]). The same increase in reporter gene expression was observed for additional leucine and glutamic acid variants (Figure 5).

4. Discussion

In this study we demonstrate for the first time the functional expression of MC4R in yeasts. We used this expression system to explore the importance of amino acid residue at position 126 of human MC4R. Our results show that wt MC4R is activated in yeast cells by at least three different ligands: the short natural peptides αMSH and ACTH, and small molecule agonist THIQ. αMSH and ACTH displayed pharmacological properties comparable to MC4R expressed in mammalian cells. However, the potency of activation in yeasts was 30-40 times lower compared to mammalian cells (Table 1). A similar difference in ligand affinities between two expression systems has been observed also for adenosine A2b receptors [27,36]. One of the main differences between mammalian and yeast expression systems is the presence of the polysaccharide-rich yeast cell wall that has to be crossed by ligands in order to reach the receptors located in a plasma membrane. Our results show no relative difference in activation potency depending on the ligand size. THIQ, which is significantly smaller (Fw=589.169), actually has a lower affinity than peptide ligands and even the 39-amino acid peptide ACTH (Fw=4541.11) is capable of migrating through the yeast cell wall and activating the MC4R. It is reported that THIQ has approximately the same affinity for MC4R as αMSH [24,37]. The lower activity in yeasts may be explained by instability of THIQ in yeast culture where the activation process takes a significantly longer time when compared to the classical cAMP experiments. It cannot be excluded that THIQ may interact with the yeast polisaccharide-rich cell wall resulting in decreased affinity.

Yeast expression systems have been used to select the functionally active variants from G-protein coupled receptor libraries [26,38], or to isolate constitutively active receptors [39]. However, so far the GPCRs that bind small molecules as agonists have usually been explored [27,30,31]. For the analysis of GPCRs, which bind larger peptides, co-expression of ligand and receptor in the same yeast cells has been used [26,29]. The extracellular activation of the V2 vasopressin receptor with the 9-amino acid vasopressin peptide has been reported for the peptide binding GPCRs in yeast cells [28].

MC4R variant	EC_{50}±SEM (nM)[a]
GAC wt	269.0±28.0
GAC c11*	535.2±15.3
GAC c18*	346.0±44.3
GAC c24*	497.4±12.1
GAC c27*	563.1±19.0

Table 3. β-galactosidase production in response to αMSH induced activation of different MC4R clones with aspartic acid at position 126.

**c11, c18, c24, c27 represent different clone id number*
a- calculated from β-galactosidase production levels in S. cerevisiae

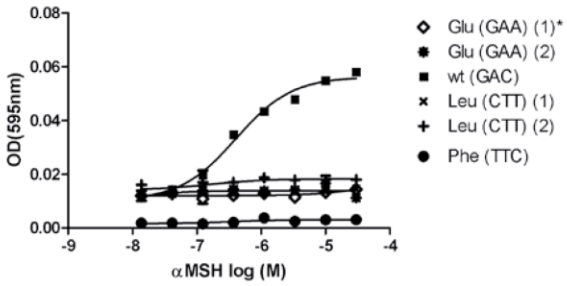

Figure 5. MC4R activation curves of individual MC4R codon variants after isolation and retransformation in *S. cerevisiae*. * - Amino acid (codon) (isolated clone No.).

Aspartic acid at position 126 is shown to be important for MC4R activity in a number of mutagenesis studies [18,24,25]. Typically, the neutral amino acid, e.g. alanine [19], is used for the substitution of the original amino acid during mutagenesis. Such an approach may lead to false results if the evaluation of the role of specific amino acid in the protein structure is considered. Aspartic acid at position 126 thus has been substituted with alanine and asparagine in the previously reported studies. The mutagenised libraries give an opportunity to increase the number of variants included in the functional analysis. In this study we were able to establish the functional consequences of 9 amino acids, representing residues with different chemical properties, at position 126 of the MC4 receptor.

During the functional activation of the obtained MC4R clones, only the variant with the aspartic acid displayed functional activity. Interestingly, the MC4R variant with the glutamic acid was functionally inactive. Both these amino acids have acidic chemical characteristics that are considered to be important for binding the pharmacophore of natural ligands at this position of the MC4R [23]. Although aspartic acid and glutamic acid are very similar structurally, glutamic acid

has a longer carbohydrate tail with a –COOH terminus. We have shown that even a single –CH group, which distinguishes aspartic and glutamic acids, is sufficient to disarrange the binding pocket of the melanocortin pharmacophore and functionally inactivate the MC4R.

MC4R variants with glutamic acid and leucine at position 126 had a higher background activity (Figure 4a) than other mutants, even after repeated plasmid isolation and retransformation. It is possible that the receptor variants with neutral, aliphatic or acidic amino acid substitution have a higher constitutive activity, and D126 of the MC4R is not only crucial for the ligand binding but is also responsible for the background constitutive activity of the receptor. The constitutive activity of the MC4R has been previously demonstrated [40].

In conclusion, we have shown for the first time the functional expression of the MC4R in *Saccharomyces cerevisidae* and have used it for functional characterization of the randomized MC4R library. In addition to the previously known results on substitution of aspartic acid at position 126, we show that none of the other, previously not tested, amino acid substitutions obtained from the randomized MC4R gene library was functionally active, further confirming the crucial role of aspartic acid in this position for the MC4R activity.

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3.2 Single nucleotide polymorphisms of the purinergic 1 receptor are not associated with myocardial infarction in a Latvian population

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Single nucleotide polymorphisms of the purinergic 1 receptor are not associated with myocardial infarction in a Latvian population

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Abstract The purinergic 1 receptor (P2RY1) has been implicated in development of heart disease and in individual pharmacodynamic response to anticoagulant therapies. However, the association of polymorphisms in the P2RY1 gene with myocardial infarction (MI), and its associated conditions, has yet to be reported in the literature. We evaluated seven known SNPs in P2RY1 for association with MI in a Latvian population. Seven independent parameters that are related to MI [body mass index (BMI), type 2 diabetes (T2D), angina pectoris, hypertension, hyperlipidemia, atrial fibrillation and heart failure] were investigated. No significant association with MI was observed for any of the polymorphisms. Those SNPs for which the *P* value was close to significance were located in coding or promoter regions. Intriguingly, carriers of the minor allele in the P2RY1 gene locus showed a tendency towards higher onset age for MI, suggesting a possible protective effect of these SNPs against MI or their contribution in progression as opposed to onset. Finally, a linkage disequilibrium (LD) plot was generated for these polymorphisms in the Latvian population. The results of this study suggest that the role of P2RY1 in individuals from Latvian population is likely to be principally involved in platelet aggregation and thromboembolic diseases, and not as a significant contributing factor to the global metabolic syndrome.

Keywords Purinergic receptor · Myocardial infarction · Genetic association · Heart diseases

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Introduction

The purinergic 1 receptor (P2RY1) is a member of the plasma membrane G protein-coupled purinergic receptor family. Expressed in a broad range of cell types [1–4], these receptors have been shown to regulate many crucial physiological functions, such as blood coagulation, neuronal signalling, muscular functions and inflammation [5–8]. P2RY1 acts in conjunction with the purinergic 12 receptor (P2RY12) to activate platelet-expressed αIIbβ3 integrin receptor, which mediates activation of platelet aggregation [9]. Following any blood vessel injury,

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activated platelets bind to von Willebrand factor [10] and release specific, primed granules upon recognition of collagen molecules in the vessel wall; these secreted granules then discharge their contents of hormones and adenosine di- and triphosphates (ADP and ATP, respectively) into the blood circulation [11]. The liberated ADP binds to P2RY1, which in turn induces activation of the G protein's Gαq subunit. Interaction of Gαq with phospholipase C-β mobilizes intracellular calcium ions, changing platelet cell shape and initiating aggregation [12]. Meanwhile, binding of ADP to P2RY12 prompts the Gαi subunit to reduce the intracellular concentration of cyclic adenosine monophosphate (cAMP); the subsequent increase in vasodilator-stimulated phosphoprotein and other enzymes ultimately leads to αIIbβ3 integrin receptor activation [9, 13, 14].

P2RY12 has become the major molecular target of antiplatelet pharmaceuticals (clopidogrel, ticlopidine) based upon its role in thrombus formation. By blocking the binding site of P2RY12, and impeding its downstream activation of the pathway leading to coagulation, patients who have undergone cardiovascular surgery, like stenting, experience better long-term outcome [15, 16]. The P2RY12 gene has been extensively studied to determine the particular genotypes associated with different cardiac phenotypes [17–21]. Several reports have implicated P2RY1 to be associated with coronary dysfunctions and considered it as a target for antiplatelet therapies [22, 23]. However, there is no established use of a P2RY1 antagonist in clinical practice.

A synonymous single nucleotide polymorphism (SNP), rs701265, was identified in the coding region of P2RY1 and appeared to be associated with higher platelet reactivity to ADP in a study of healthy adults [24]. This association was not replicated in a subsequent study of a similar, but smaller population [25]. The rs701265 SNP does not play a detectable role in platelet response to clopidogrel [26, 27]. Although two studies were unable to find any association with the aspirin-resistant phenotype, [26, 28], rs701265 was significantly correlated with platelet responsiveness to aspirin in myocardial infarction (MI) patients after percutaneous coronary intervention [29].

Another well-studied polymorphism in the P2RY1 gene is rs1065776, which is located in the upstream non-coding sequence. This SNP has been associated with aspirin resistance in both Caucasian and Chinese populations [28–30]. Moreover, significant effects of rs1065776 on platelet responses to collagen and ADP were observed in healthy adults [31]. A large clinical cohort study on seven P2RY1 SNPs (rs701265 and rs1065776, and including rs2579133, rs17451266, rs9289876, rs16864613, rs12497578) found no association with the clopidogrel analog ticagreol treatment and platelet reactivity [32].

Still, no study on the possible impact of P2RY1 SNPs on myocardial disease outcome has been reported in the literature. We describe here our investigations into the role of selected tagSNPs on MI, using groups of healthy and diseased individuals from Latvian population.

Materials and methods

Study group

We used the STrengthening the REporting of Genetic Associations (STREGA) guidelines [33] to design our study group and methods of association analysis. Case–control study groups were selected from the Latvian Genome Data Base (LGDB) national biobank of health and genetic information collected for adult residents of Latvia (over 18 years old). All recorded data relating to health status had been affirmed by physicians using International Classification of Diseases (ICD)-10 codes. Data for anthropometric measurements (including weight and stature) had been obtained by direct measurement. Demographic data, such as ethnic, social, and environmental information, and familial health status had been obtained by questionnaire-based interview. LGDB participant recruitment is ongoing and carried out by medical personnel in hospitals or general clinical practices. No specific health condition is required for involvement. Written confirmed consent had been acquired from all LGDB participants. The study protocol was approved by the Central Medical Ethics Committee of Latvia.

The study group for this investigation was selected from among the entire pool of LGDB participants ($n = 11{,}342$) recruited from 2003 to 2009. First, we excluded all individuals with cancer (ICD: from C00 to C97), renal conditions (ICD10: from N00 to N99) or rare diseases (detailed list available upon request), to avoid overwhelming confounding influences on the analysis. Second, any remaining individuals with incomplete phenotype data were removed. After this selection, patients with MI diagnosis were selected; MI diagnoses were verified by professional cardiologists according to symptomology at presentation, electrocardiogram results and biomarkers in blood. A total of 1,781 individuals comprised the final MI study group (females, $n = 536$ and males, $n = 1{,}245$). To select the healthy study group (controls), the initial pool of LGDB participants were filtered to remove all subjects with chronic diseases (detailed list available upon request) and heart and coronary disease conditions (ICD10: from I20 to I70), leaving 1,725 healthy adults. For analysis, 263 females and 592 males were randomly selected from the MI group and 855 controls were randomly selected from healthy group, maintaining the same sex proportion as in the case group. Seven of the control males were later excluded due to discovery of missing phenotype information.

Smoking status was assigned according to answers obtained by the original questionnaire. Individuals were assigned to the smoking group if they had smoked at least three cigarettes per day for the previous 1 year. Those individuals who reported a history of regular smoking, but had quit, were also assigned to the smoking group. The level of routine physical activity was also assigned according to the history of reported physical activity. Alcohol consumption was grouped as consumer or non-consumer. The non-consuming individuals were identified as those who reported no alcohol consumption at all along with those who consumed alcohol less than once a week and less than 1.5 units (U) of alcohol total.

SNP selection

Known SNPs were selected for analysis with the aim of optimally covering the common genetic variation of the P2RY1 gene in humans. The international HapMap project database (Rel#24; www.hapmap.org) was mined to obtain information on SNP frequencies. Tag SNPs were selected by using HaploView software (version 4.2; www.broadinstitute.org) [34–36] set to capture all SNPs with a minor allele frequency (MAF) of >0.01 and an r^2 of >0.8. The SNPs rs1439009 and rs701265 were selected for force inclusion in the tagger software based on previous reports of association with perturbed platelet reactivity or cardiac dysfunctions. The calculated tagSNPs were rs2579133, rs17451266, rs9289876, rs1439009, rs701265, and rs12497578. rs1439009 was in strong linkage disequilibrium (LD) with rs1439026, rs16864614, rs16864613, and rs16864605, and genotyping of rs701265 encompassed rs1371097. Thus, the use of only six tagSNPs provided coverage of 11 SNPs in the P2RY1 gene locus and took into account possible imputation of all these SNPs.

The rs1065776 SNP was separately chosen for inclusion in our analysis, based upon literature data. No allele frequency information was available for this SNP in the HapMap database.

PCR amplification and genotyping

Stock DNA samples acquired from the biobank were diluted into 96-well PCR plates (28 ng per well) using the Freedom Evo robotic workstation (Tecan, Männedorf, Switzerland). PCR primers for amplification of genomic DNA containing SNPs of interest were designed by modified Primer3 program (http://primer3.sourceforge.net/) and synthesized by Metabion (Martinsried, Germany). The primer sequences are listed in Online Resource 1. Amplification was carried out by standard polymerase chain reaction (PCR), using a reaction mix containing 1 mM DB buffer, 2.5 mM $MgCl_2$, 0.5 U HotFire polymerase, 0.2 mM dNTP mix (all from SolisBioDyne, Tartu, Estonia), 0.3 mM each primer, and 28 ng template DNA. PCR was carried out on a Veriti96 ThermalCycler (Applied Biosystems, USA) using the following conditions: denaturation at 95°C for 5 min; 40 cycles of amplification at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and final extension at 72°C for 5 min. Products were confirmed by visualization on agarose gel after electrophoresis; dephosphorylation of unused dNTP in the reactions was carried out by addition of shrimp alkaline phosphatase (Fermentas, Lithuania) according to the manufacturer's protocol.

For minisequencing, the CalcDalton program [37] was used to design special primers that contained a biotin cap on 5′-end and a photolinker cleavage site (synthesized by Biotez, Germany). The primer sequences are listed in Online Resource 1. The minisequencing reaction contained 0.5 mM 10× C buffer, 1.25 mM $MgCl_2$, 1 U TermiPol DNA polymerase (all from SolisBioDyne), 0.2 mM ddNTPs (Fermentas, Lithuania), 1 mM each primer, and 5 µl of PCR product. Minisequencing conditions were: denaturation at 95°C for 2 min; 99 cycles of amplification at 95°C for 10 s, 55°C for 10 s, and 72°C for 10 s; and final extension at 72°C for 5 min. Minisequencing products were precipitated on streptavidin-coated 384-well plates (Biotez) and subjected to UV exposure for 15 min.

Time-of-flight mass spectrometry (TOF-MS) analysis

An aliquot of 1 µl of purified DNA minisequencing product was diluted in 1 µl of 3–hydroxypicolinic acid matrix (70 and 40 mM diammonium hydrogen citrate), spotted onto an MTP AnchorChip 400/384TF (Bruker Daltonik, Germany), and air-dried. Matrix-assisted laser desorption/ionization (MALDI)-TOF MS analysis was carried out on a Autoflex MS (Bruker Daltonik, Germany) using a maximum of 40 nitrogen laser shots with frequency of 16.7 Hz for each sample spot. The low molecular weight oligonucleotide calibration standard (1,000–4,000 Da; Bruker Daltonik) was used for mass calibration. For genotyping of rs2579133 and rs701265: 4,000–5,000 Da was used; for rs1439009, rs1065776 and rs12497578: 1300–3600 Da was used; for rs17451266 and rs9289876: 1,900–4,200 Da was used. Results were analysed by using the Genotools SNP Manager software (Bruker Daltonik, Germany) followed by manual verification of findings.

Statistical analysis

Statistical analysis was performed using Plink 1.06 software [38] (http://pngu.mgh.harvard.edu/purcell/plink/). Testing for deviation from Hardy–Weinberg equilibrium was performed as implemented in Plink 1.06. The χ^2 and Student's

t test were used to compare characteristics between case and control groups. Possible associations were evaluated by logistic regression. The logistic regression assuming additive model of inheritance was employed to adjust the analysis for other non-genetic influences. The cofactors of sex, age, BMI, smoking, alcohol consumption and physical activity were included in logistic regression models. To test the impact of SNPs on MI depending on obesity, the study group was divided into BMI < 30 (normal weight) and BMI > 30 (obese). Thus, for each group, the calculation of association with MI was carried out using logistic regression and the same cofactors, with the exception of BMI. SNPs were also tested for association with disease states—type 2 diabetes (T2D), angina pectoris, hypertension, hyperlipidemia, atrial fibrillation, heart failure and obesity (BMI > 30). Haplotype association and estimation of haplotype frequencies were performed using Plink 1.06 [38]. Linear regression was used for analysis of SNPs association with age of MI onset. LD plot was generated using the HaploView software 4.2. [34–36]. Power calculations were performed using Quanto v.1.2.3 [39] (http://hydra.usc.edu/gxe/). The allele frequencies estimated in our study were applied. Population risk for MI was set at 0.01 and α was set at 0.05. An additive mode of inheritance was assumed for power calculations.

Results

Characteristics of the MI and healthy study groups are presented in Table 1. Comparative analysis demonstrated that significant differences existed between the case and control groups in mean age, mean body mass index (BMI), T2D, hypertension, alcohol consumption and hyperlipidemia incidence (Table 1). A less significant difference was observed in smoking status ($P = 0.04$) between the two groups, and no difference was observed for physical activity.

All 1,703 subjects were genotyped using MALDI-TOF MS. The genotyping success of each polymorphism is presented in Table 2. From all SNPs genotyped, only the rs17451266 showed deviation from Hardy–Weinberg equilibrium. The lowest MAF was represented by rs2579133 rare allele A (0.3%), and the highest MAF was

Table 1 Phenotypic characteristics of study group

Characteristic	Cases (855)	Controls (848)	*P* value
Female, *n* (%)	263 (31%)	268 (32%)	0.877
Male, *n* (%)	592 (69%)	580 (68%)	0.759
Mean age, (±SD) years	62.0 ± 11.3	54.5 ± 12.2	<0.0001
Mean BMI, (±SD) kg/m^2	28.62 ± 4.58	26.97 ± 4.42	<0.0001
Myocardial infarction	855 (100%)	0	–
Type 2 diabetes, *n* (%)	126 (15%)	20 (2%)	<0.0001
Smoking, *n* (%)	508 (59%)	456 (54%)	0.042
Physically active, *n* (%)	138 (16%)	147 (17%)	0.623
Alcohol consumption	355 (42%)[a]	533 (66%)[a]	<0.0001
Angina pectoris	258 (30%)	0	–
Hypertension	464 (54%)	102 (12%)	<0.0001
Hyperlipidemia	289 (34%)	9 (1%)	<0.0001
Atrial fibrilation	69 (8%)	0	–
Heart failure	493 (58%)	0	–

[a] Alcohol consumption information were available for 803 controls and 836 cases

Table 2 Characteristics of SNPs genotyped in the P2RY1 locus

SNP no.	rs code	Genome position	Common allele	Rare allele	MAF	Genotyping success (%)	H–W test *P* value
1	2579133	149939559	G	A	0.003	98.6	1
2	17451266	149941013	G	T	0.123	98.1	0.0013
3	9289876	149941500	A	T	0.03	95.7	1
4	1439009	149942344	A	T	0.026	97.9	0.1097
5	1065776	149944147	C	T	0.024	96.1	0.0884
6	701265	149944876	A	G	0.121	98.6	1
7	12497578	149946992	C	G	0.095	99.1	0.8453

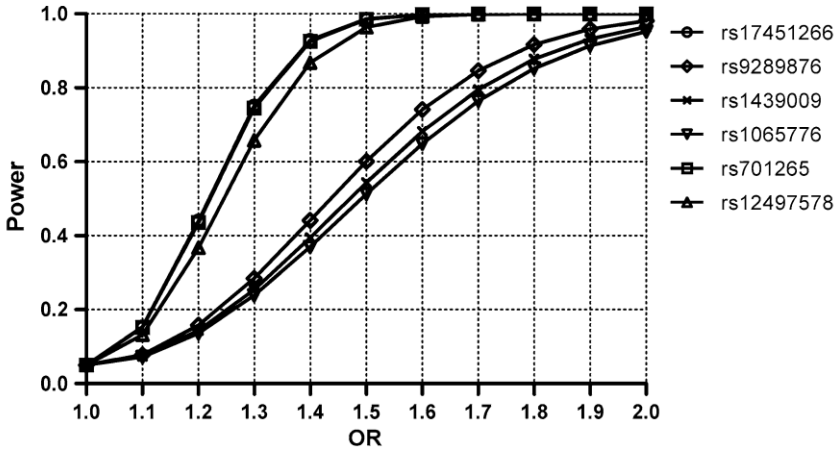

Fig. 1 Power to detect genetic association ORs for six analysed SNPs. The power calculations assumed a multiplicative mode of inheritance. Allele frequencies determined in our study were used; α was set at 0.05

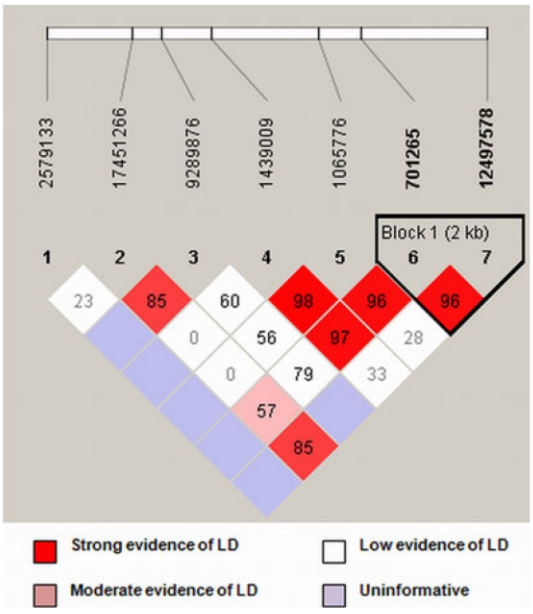

Fig. 2 LD plot of the P2RY1 region. LD was determined considering all 1,703 subjects. The pairwise D' values are presented in each *box*

shared by rs701265 G (12%) and rs17451266 T (12%) alleles (Table 2). Power to detect odds ratios (ORs) for six of the SNP's associations with MI was calculated (Fig. 1). rs2579133 was excluded from this analysis due to its exceedingly low MAF; we were only able to detect an OR of 3.65 with 80% power for this SNP.

Linkage disequilibrium calculations using the genotyping data revealed a strong LD occurred between rs701265 and rs12497578, as well as between rs1439009, rs1065776 and rs701265 with D' values over 95 (Fig. 2).

The results of the logistic regression analysis for the seven SNPs association with MI are shown in Table 3 (adjusted for sex, age, BMI, smoking, alcohol consumption and physical activity). We observed no statistically significant association with MI for any of the SNPs evaluated in our study.

Other available phenotypes were tested for association; again, no significant association was observed for any of the SNPs with T2D, angina pectoris, hypertension, hyperlipidemia, atrial fibrillation and heart failure ($P > 0.05$). This finding was also true when cases or controls were

Table 3 Association of MI with seven P2RY1 SNPs

SNP	Number of subjects		*P* value*	OR [95% CI]*
	Cases	Controls		
2579133	0/5/846	0/4/825	0.579	0.60 [0.10–3.59]
17451266	20/162/660	24/160/644	0.370	0.89 [0.69–1.15]
9289876	3/49/774	0/44/760	0.734	0.91 [0.53–1.56]
1439009	0/44/801	2/40/782	0.640	0.87 [0.50–1.54]
1065776	0/38/790	2/37/770	0.453	0.80 [0.44–1.44]
701265	13/181/659	12/176/639	0.345	0.88 [0.68–1.15]
12497578	7/144/697	7/150/683	0.315	0.86 [0.65–1.15]

* *P* value and OR were calculated using logistic regression adjusted for sex, age, BMI, smoking, alcohol consumption and physical activity

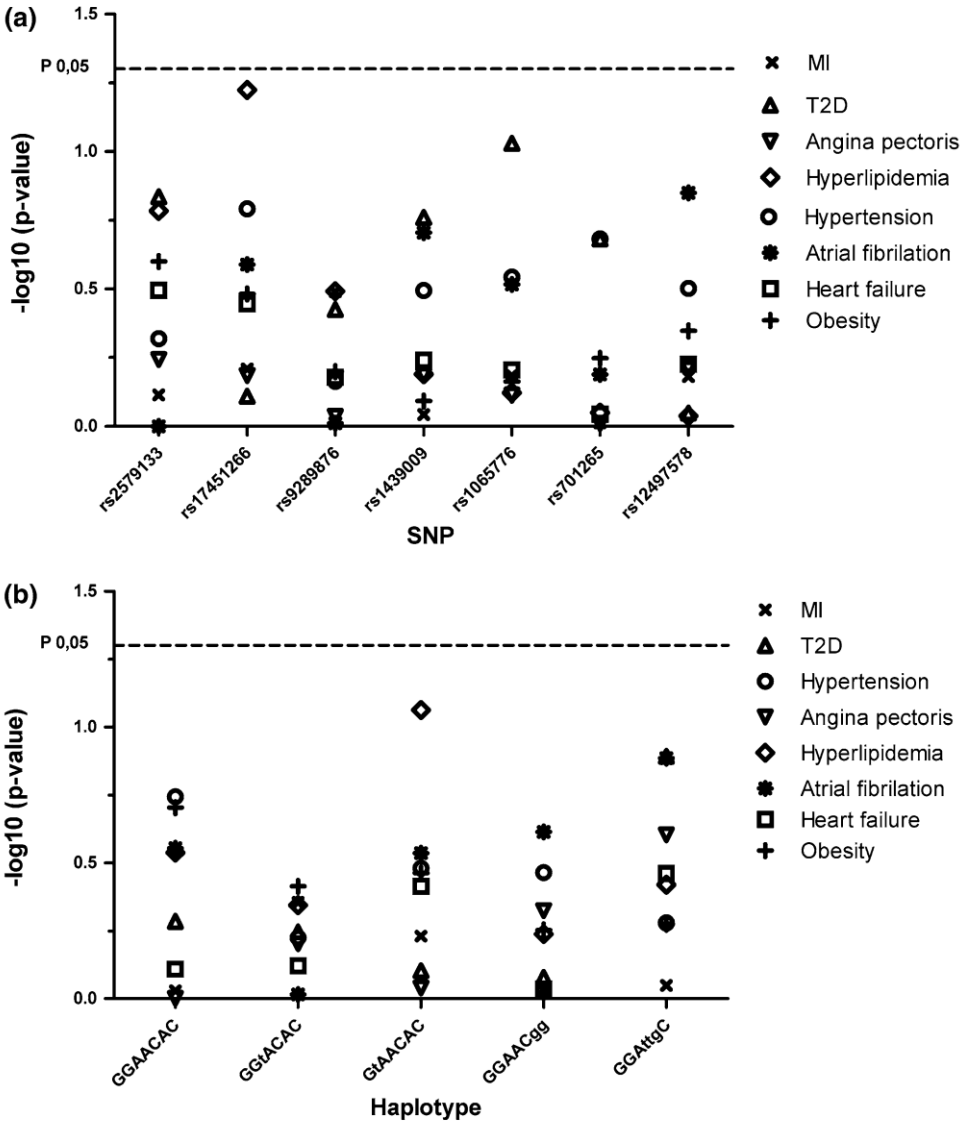

Fig. 3 SNPs (**a**) and haplotype (**b**) associations with phenotypes. Significance values were transformed as negative logarithm of *P* value from an unadjusted logistic regression analysis. *P* values were calculated by dividing the whole group (cases or controls) by the phenotype prevalence. The major allele is indicated by *uppercase letters*, and the minor allele by *lowercase*

evaluated separately (where appropriate) (Fig. 3a). Five haplotypes were derived from the seven SNPs with allele frequency over 1%: GGAACAC (73%), GGtACAC (3%), GtAACAC (12%), GGAACgg (9%), GGAttgC (2%) (upper-case letters represent the major allele and lower-case letters the minor allele). None of these haplotypes were significantly associated with MI or any other phenotype (Fig. 3b, Online Resource 2).

Weak evidence of association with MI was found for the obese study group (BMI > 30) and SNPs rs9289876, rs1439009, rs1065776 ($P = 0.064$, $= 0.078$, $= 0.087$, respectively) (Online Resource 3). Haplotype GGAttgC had a *P* value very near the threshold of significance (Online Resource 3); this was lost upon adjustment for sex and age (Online Resource 4).

Data on the age of MI onset were available for 677 of the MI subjects. We found a slight association between the rs1065776 T allele and later disease onset. Calculation of mean age of MI onset for different genotypes revealed that there might be a tendency for later onset of MI in those subjects who carry the minor alleles (Online Resource 5).

Discussion

In this study we tested, for the first time, the role of P2RY1 polymorphisms for their associations with MI, while considering seven important comorbidities related to MI (BMI, T2D, angina pectoris, hypertension, hyperlipidemia, atrial fibrillation and heart failure). Statistical analysis of seven known SNPs in the P2RY1 gene locus of 855 MI patients and 848 control group subjects demonstrated that there was no significant association with MI for any. Moreover, we did not observe any statistical significance to the other phenotypes examined, even though they are important for cardiovascular disease development.

Previous functional studies have linked P2RY1 to platelet aggregation, which plays an important role in thromboembolic disease. It has been suggested that this role may contribute to the formation of occlusive thrombi that can lead to MI and heart failure. Interestingly, the P2RY1 is characterized as having a broad cell type expression pattern in human tissues, including in brain, heart, skeletal muscle, kidneys, adipose tissue and pancreas [1, 2, 16, 22]. However, the different physiological functions that the P2RY1 mediates in each of these tissues have yet to be fully elucidated. This has prompted speculation that the P2RY1 may play a general role in atherosclerosis development, which itself is considered a major risk factor for angina pectoris [16]. The P2RY1 expression in various areas of brain and in adipose tissue raise the possibility that it might be able to influence regulation of lipid metabolism [1, 22]. Likewise, the expression of P2RY1 in the pancreas suggests a possible role related to T2D development [22]. Functional studies of P2RY11 have demonstrated that hetero-oligomerization of P2RY1 and P2RY11 is crucial for P2RY11 internalization and signalling [40]; but the exact physiological functions of P2RY11 remain poorly understood. The P2RY1 might have the greater influence on resulting metabolic traits influenced by the P2RY11-P2RY1-mediated signalling. Previously published results from human genetic research have assessed effects of polymorphisms on physiological features of ADP-induced platelet response and aspirin or clopidogrel resistance [24–32], all of which are crucial features that impact the success of MI treatment. However, none of these studies were able to detect significance of genetic variation in progression of MI or other related heart conditions. Moreover, MI development is a complex process and many different physiological traits have been defined as risk factors. These factors may also interact with one other, making it very difficult to determine the exact profile of MI causative factors. For example, hyperlipidemia contributes to atherosclerosis of blood vessel walls and subsequently causes angina pectoris; angina pectoris is an important clinical indicator of forthcoming MI. Hypertension is another factor that affects the blood vessel endothelium; high pressure on the walls of the blood vessels then causes micro-damage, which induces inflammation and the plaque formation that can lead to MI. Finally, high BMI correlates strongly with hyperlipidemia, hypertension and diabetes development; patients suffering from diabetes frequently experience cardiomyopathies and other heart dysfunctions that are known to contribute to MI [41]. Many other cardiac-related complications, such as heart failure and atrial fibrillation, are often diagnosed in MI patients, and likely influence MI disease outcome. The prevalence and broad range of comorbidities diagnosed in MI patients highlight the need for studies to appropriately adjust for various confounding factors. The negative results that we obtained for P2RY1 SNPs associations with MI suggest that the role of this particular gene is likely to be limited to platelet aggregation and thromboembolic diseases, and not an important contributor to the global metabolic syndrome. However, many other factors that are involved in blood coagulation with MI, coronary artery disease or other related conditions have also been studied in the same manner [42–44].

In our study, we did observe a positive correlation for all examined P2RY1 tagSNPs, except rs9289876, with late-onset MI. It is possible that genetic variation of the P2RY1 gene locus may influence the progression of established heart disease in a manner independent of the other phenotypes that were evaluated in this study. Further research with greater statistical power is needed to investigate this hypothesis.

Previous studies have mainly focused on two SNPs in the P2RY1 coding (rs701265) or promoter region (rs1065776) [24, 25, 27–29]. Here, we extended our analysis to characterize seven known SNPs in order to attain more complete coverage of the P2RY1 gene locus. Six of the polymorphisms we examined were selected according to tagSNP analysis of HapMap data and in consideration of the relative LD of other SNPs in the P2RY1 locus. In this way, we were able to generate a detailed LD map of P2RY1. While there is currently no data in HapMap regarding the frequency of rs1065776, several studies in the literature have focused on this particular SNP [28, 29, 32]; thus, we included this SNP in our study and performed the first LD analysis for this polymorphism. We found strong LD between rs1439009 and rs701265, rs701265 and rs12497578, which was in agreement with the HapMap data of Caucasians of central European (CEU) ancestry. rs1065776 was found to be in strong LD with the two closest neighbouring SNPs, rs1439009 and rs701265; moreover, rs17451266 and rs9289876 proved to be in LD with rs1065776, having a D' value of 85. Surprisingly, rs17451266 and rs12497578 also had D' values of 85, even though they are separated by almost 6 kb. The lowest P values for each of the phenotypes evaluated corresponded to SNPs located in the gene's promoter region (rs9289876, rs1439009 and rs1065776), two of which (rs1439009 and rs1065776) were in strong LD with rs701265 in the P2RY1 coding sequence. Collectively, these findings can serve as a valuable basis for SNP selection in future investigations of the P2RY1 locus in relation to other disease phenotypes.

Overall, the minor allele frequencies of these SNPs were similar to those previously published. However, rs2579133 exhibited a more than threefold lower frequency in the Latvian population examined in our study, as compared to

data that has been reported in the NCBI HapMap database or in the related research literature [32]. rs1439009 and rs1065776 also exhibited lower frequencies in Latvians than those reported in NCBI HapMap and [28, 29, 32]. Since most of the published studies have been performed on smaller population groups (<200) [25–27, 32], this variation in allele frequencies is not surprising.

As ours is the first study to describe the possible role of sequence variations in the P2RY1 gene locus to development of MI, there is no previous data on effect sizes of the SNPs we examined. In order to determine the potential limitations of our study, we estimated the power to detect association assuming our sample size. Our study was sufficiently powered to detect OR for the six HapMap-reported SNP's association with MI from 1.3 to 1.7 with 80% power (Fig. 1). For the seventh SNP examined, rs2579133, the low MAF necessitated a significantly larger sample size to facilitate accurate detection of small effect size.

Thus, while we were able to fully describe the P2RY1 gene alleles in relation to MI and seven related phenotypes, we were unable to completely exclude the association of analysed SNPs with MI having very small effects on disease risk.

In conclusion, we have demonstrated that known SNPs in the P2RY1 gene locus are not associated with MI, BMI, T2D, angina pectoris, hypertension, hyperlipidemia, atrial fibrillation or heart failure in the Latvian population. However, a tendency towards higher MI onset age was observed in individuals carrying minor alleles of P2RY1 polymorphisms, suggesting that P2RY1 may contribute to progression of established heart disease in this population or might mediate a protective effect against MI. Finally, the detailed LD map generated in this study for the P2RY1 locus in the Latvian population can act as a guide tagSNP selection in future studies.

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Online Resource 1 **Primers used in this study**

rs code	Primers for amplification
rs2579133	Fw - ACG TTG GAT GCT TTA TCT CAT AAA TCA GGG TCT C Rs - ACG TTG GAT GAG AAT GGG AAA ATC ATT GG
rs17451266[a] rs9289876	Fw - ACG TTG GAT GAT TAA CAT GGA ATG CTT AGG G Rs - ACG TTG GAT GCT GAC TTC ACT CAG AAG TTT GTT
rs1439009	Fw - ACG TTG GAT GGT GCT AGA TTC CAT TCT TGT G Rs - ACG TTG GAT GAC GGT TAT AAT TCC AAA GTG AC
rs1065776	Fw - ACG TTG GAT GTC TAA GGT AGG GAG GAG GAA Rs - ACG TTG GAT GTT TGA ACG ACG AGG AGA C
rs701265	Fw - ACG TTG GAT GGC TTT GAT TTA CAA AGA TCT GG Rs - ACG TTG GAT GAC ATG GAA AGG GAT GTA AGA
rs12497578	Fw - ACG TTG GAT GGC CTA TGT AGA TTG AGG ATT TG Rs - ACG TTG GAT GAA TCA CTG CTT TGG AAG GT
rs code	**Primers for minisequencing**[b]
rs2579133	bioTCT TAC CTA (L)CA GCT CCC AAC TTC
rs17451266	bio TT ATT CCC ACA AGG C(L)A AAT TTT
rs9289876	bioGA AAT GTA CAC TT(L) GTC CAG ATA CCA
rs1439009	bioA GAA AAC GAG TTT GTC (L)GT AAA TGT
rs1065776	bioCCA GGA C(L)A ACC CGG ACC
rs701265	bioC AGC AAA AAC (L)GT CAG TAC AAT GAT
rs12497578	bioT CTT CAT TGA TCT TAG (L)GG CCA

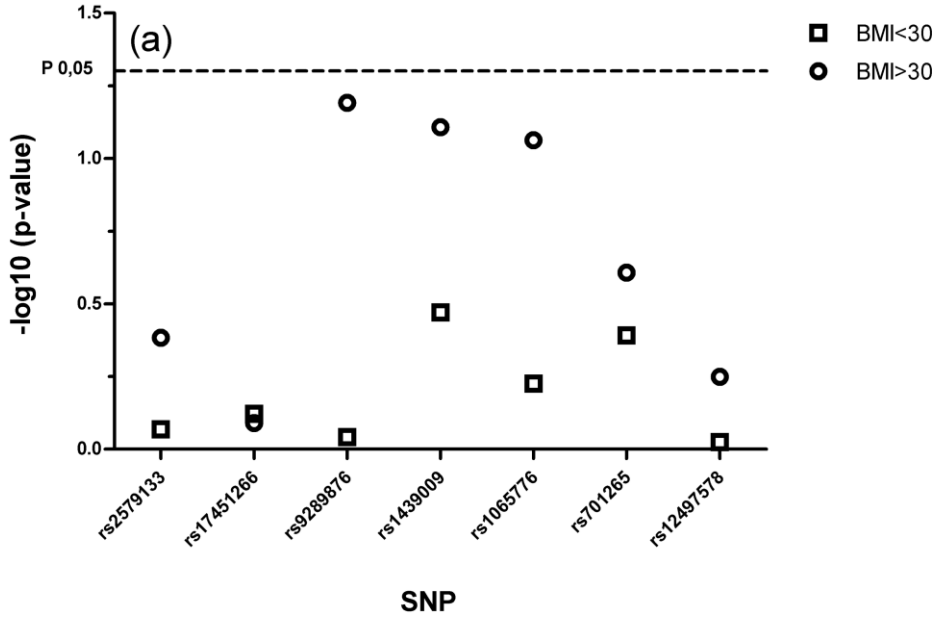
[a] for both SNPs the same primers were used.
[b] bio - indicate biotin cap; (L) - photolinker cleavage site.

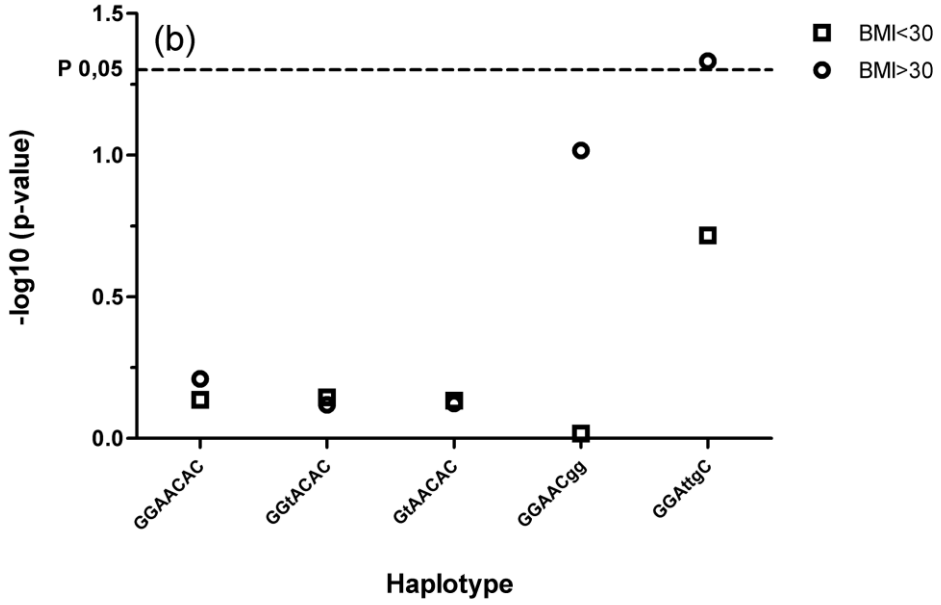
Online Resource 2 **The distribution of haplotype frequencies in MI patients and the control group, including haplotype association with MI**

Haplotype No.	Haplotype	Frequency in MI patients	Frequency in controls	Haplotype *p*-value*
1	GGAACAC[a]	72.9%	72.1%	0.935
2	GGtACAC	3.1%	2.7%	0.442
3	GtAACAC	11.4%	12.1%	0.588
4	GGAACgg	9.1%	9.1%	0.925
5	GGAttgC	1.9%	1.9%	0.894

**p*-value was calculated using haplotype association test as implemented in Plink 1.06 (Purcell *et al.*, 2007).
[a] Uppercase letters represent the major allele, lowercase letters the minor allele.

Online Resource 3 **Association of SNPs (a) and haplotype (b) with MI in study groups with BMI<30 (normal weight) and BMI>30 (obese). Significance values were transformed as negative logarithm of *p*-value from an unadjusted logistic regression analysis. The major allele is indicated by uppercase letters, and the minor allele by lowercase.**





Online Resource 4 **Association of MI with seven *P2RY1* SNPs in BMI<30 (normal weight) and BMI>30 (obese) study groups**

SNP	BMI<30 *p*-value*	OR [95% CI]*	BMI>30 *p*-value*	OR [95% CI]*
2579133	0.396	0.37 [0.04 - 3.65]	-[a]	-
17451266	0.919	0.98 [0.73 - 1.33]	0.376	0.80 [0.49 - 1.31]
9289876	0.472	0.79 [0.4 - 1.51]	0.470	1.53 [0.48 - 4.88]
1439009	0.594	0.83 [0.42 - 1.645]	0.747	0.85 [0.31 - 2.32]
1065776	0.307	0.69 [0.34 - 1.41]	0.955	0.97 [0.34 - 2.80]
701265	0.923	0.98 [0.72 - 1.35]	0.193	0.72 [0.45 - 1.18]
12497578	0.975	1.00 [0.71- 1.42]	0.129	0.65 [0.38 - 1.13]

**p*-value and OR were calculated using logistic regression adjusted for sex, age, smoking, alcohol consumption and physical activity.
[a] No minor allele.

Online Resource 5 **Association of mean age of MI onset with seven *P2RY1* SNPs**

SNP No.	rs code	Allele, Mean age (count, n)			*p*-value*
1	2579133	T/T -	T/C 62.75(4)	C/C 59.89(669)	0.6305
2	17451266	T/T 61.12(17)	T/G 60.32(128)	G/G 59.8(530)	0.5459
3	9289876	A/A 64(2)	A/T 58.25(44)	T/T 60.11(621)	0.4624
4	1439009	T/T -	T/A 62.76(38)	A/A 59.63(632)	0.111
5	1065776	T/T -	T/C 63.65(34)	C/C 59.67(624)	0.05387
6	701265	G/G 61(11)	G/A 60.95(147)	A/A 59.58(517)	0.2177
7	12497578	C/C 61.83(6)	C/G 60.16(115)	G/G 59.69(550)	0.5957

**p*-value was calculated using unadjusted linear regression.

3.3 Identification and analysis of functionally important amino acids in human purinergic 12 receptor using a *Saccharomyces cerevisiae* expression system

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Identification and analysis of functionally important amino acids in human purinergic 12 receptor using a *Saccharomyces cerevisiae* expression system

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Keywords
G-protein-coupled receptors; P2Y ligand binding pocket; P2Y receptor mutagenesis; P2Y receptors; *Saccharomyces cerevisiae*

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The purinergic 12 receptor (P2Y12) is a major drug target for anticoagulant therapies, but little is known about the regions involved in ligand binding and activation of this receptor. We generated four randomized P2Y12 libraries and investigated their ligand binding characteristics. P2Y12 was expressed in a *Saccharomyces cerevisiae* model system. Four libraries were generated with randomized amino acids at positions 181, 256, 265 and 280. Mutant variants were screened for functional activity in yeast using the natural P2Y12 ligand ADP. Activation results were investigated using quantitative structure–activity relationship (QSAR) models and ligand–receptor docking. We screened four positions in P2Y12 for functional activity by substitution with amino acids with diverse physiochemical properties. This analysis revealed that positions E181, R256 and R265 alter the functional activity of P2Y12 in a specific manner. QSAR models for E181 and R256 mutant libraries strongly supported the experimental data. All substitutions of amino acid K280 were completely inactive, highlighting the crucial role of this residue in P2Y12 function. Ligand–receptor docking revealed that K280 is likely to be a key element in the ligand-binding pocket of P2Y12. The results of this study demonstrate that positions 181, 256, 265 and 280 of P2Y12 are important for the functional integrity of the receptor. Moreover, K280 appears to be a crucial feature of the P2Y12 ligand-binding pocket. These results are important for rational design of novel antiplatelet agents.

Introduction

Purinergic receptors have two structurally different subgroups, the P2X receptors that are ligand-gated ion channels, and the P2Y G protein-coupled receptors (GPCRs) [1–3]. The human genome has eight genes, coding for P2Y–P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11, which all functionally couple to the Gq subunit of G proteins to activate the phospholipase Cβ transduction pathway. However, P2Y12, P2Y13 and P2Y14 mediate their signals via Gi and inhibit adenylate cyclase. Natural agonists of these receptors are ADP, ATP, UDP, UTP and UDP-glucose [4]. P2Y12 is activated only by ADP [5]. Several unnamed receptors have considerable sequence homology with the P2Y receptors, but have yet not been shown to have nucleotide affinity [4,6–8].

P2Y12 is expressed on various human cell types including platelets, where it plays a critical role in platelet aggregation [5,8,9]. Stimulation of P2Y12 by

Abbreviations
EL, extracellular loop; GPCR, G-protein-coupled receptors; P2, purinergic receptors; P2Y1, purinergic 1 receptor; P2Y12, purinergic 12 receptor; QSAR, quantitative structure-activity relationship; TM, transmembrane.

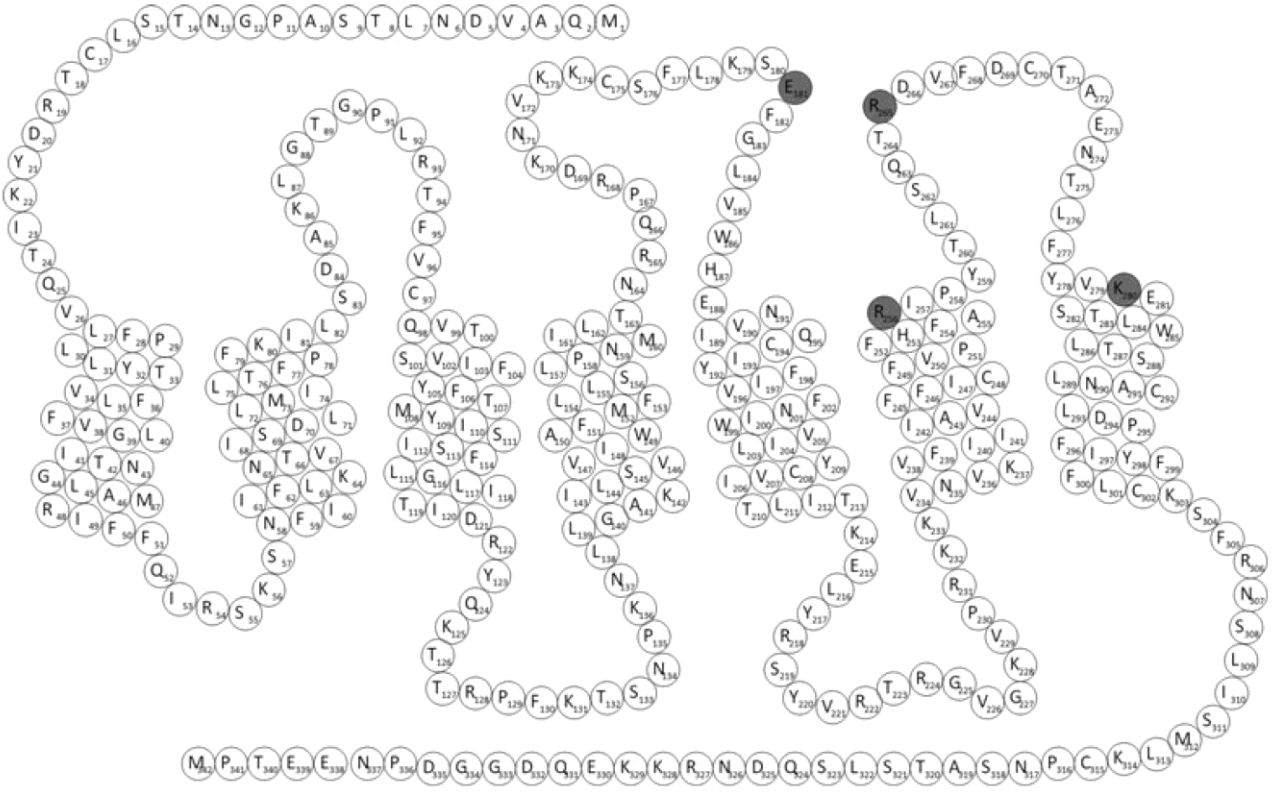

Fig. 1. Structure of P2Y12. Residues in grey were explored in this study.

the natural agonist ADP leads to αIIbβ3 integrin receptor activation, which binds fibrinogen, resulting in the formation of a blood clot [10–12]. P2Y12 is a target receptor for drugs such as ticlopidine and clopidogrel, which are frequently used prophylactically to protect against thrombosis after coronary interventions such as stenting or angioplasty [13,14].

Although P2Y12 is a major drug target, little is known about its functional domains. Few publications [15,16] have investigated P2Y12 although many functional studies have been carried out on other P2Y receptors [17–20]. The most studied is probably P2Y1, which is closely related to P2Y12 and binds the same natural agonist, ADP [5,21–23]. Overall, transmembrane (TM) 3, TM6 and TM7 domains are the most influential in signal transduction in the P2Ys. Strong evidence shows that a phosphate group in the ligand attracts polar amino acids (R, K) in these TM regions [19,20,24]. Mutagenesis studies of P2Y1 revealed that K280 and R286 are important in ligand recognition, and the corresponding regions of P2Y12, R256 and R265 influence receptor activity [15,16]. Other mutagenesis studies suggested that the R256 position is involved in ligand recognition [15] by P2Y12, but mutation of this position causes a minor decrease in receptor activity after activation with agonist [16]. Another residue described as important for the functional activity of P2Y12 is K280 [15] in TM7. This residue is conserved in all P2Ys [24] (Fig. 1).

The literature is controversial about the influence of extracellular loop (EL) 2 in ligand binding. This loop has been suggested to be involved in both nucleobase recognition [18] and directing the agonist into the binding pocket [23]. Several studies observed an impact of the acidic amino acid residues in EL2 [20,23,25], corresponding to E181 of P2Y12, but this position in P2Y12 has not been studied using mutational analysis.

In this study, we carried out an extensive characterization of four residues in P2Y12: E181, R256, R265 and K280. We generated randomized libraries of these positions in P2Y12 and found that all were important for receptor activation.

Results

P2Y12 expression in yeast

The *P2RY12* gene was cloned and expressed in the yeast *Saccharomyces cerevisiae* using strain MMY23. This strain contains a modified yeast pheromone-signalling pathway that enables cell growth after receptor activation, and a β-galactosidase reporter system that

provides a quantitative colour reaction that is dependent on receptor activation level. We used ADP, the natural agonist for P2Y12, to generate a ligand–receptor affinity curve. The EC_{50} value for activation of P2Y12 with ADP was 279 ± 17 nM, comparable with data in the literature [26], and the system was considered appropriate for further experiments.

Isolation of random P2Y12 variants

Four recombinant constructs randomized at amino acid positions 181, 256, 265 and 280 were generated to obtain randomized libraries of P2Y12. All vector constructs were sequenced to confirm library heterogeneity. Randomized libraries were transformed into yeast, which were grown on agar plates, followed by growth of selected clones in liquid medium for functional analysis. We selected and sequenced 50 colonies for positions 181, 256, and 265; and 100 colonies for position 280. Identified receptor variants are shown in Table 1. From the 50 colonies from the E181 library, 24 contained more than one amino acid variant-containing plasmid at the randomized position as indicated by several nucleotide peaks at the randomized positions in sequence chromatograms. Multiple codons were also detected for 28 clones from the R256 library, 31 clones from R265 and 19 clones from K280. In total, we isolated plasmids representing 15 different amino acid variants at position 181, 10 at position 256, 10 at position 265, and 15 at position 280.

Functional activation of recombinant P2Y12

ADP was used for all activation experiments with recombinant P2Y12 variants. In cases where different codons coded for the same amino acid, we used the variant containing the codon with the best yeast expression preference [27] (Table 1). All activation experiments were repeated at least three times, and the EC_{50} values and ligand–receptor affinity curves are displayed in Table 1 and Fig. 2, respectively. Functional activation of wild-type P2Y12 was also carried out with 2-methylthio-adenosine-5′-diphosphate, to compare these results with ADP activation and observe stability of ADP in yeast expression system (Fig. S1).

All substitution variants for the E181-randomized library were functionally active, although the E181L and E181I variants showed almost fivefold lower activity compared with wild-type P2Y12. All recombinant variants at position 256 displayed at least fivefold lower activity. Furthermore, the R256P and R256Y variants were functionally inactive. The obtained variants of the amino acid 265 library generally demonstrated decreased activity levels compared with the wild-type P2Y12, with especially low EC_{50} values for R265E, R265A, R265L and R265I. Any substitution of the 280 position with exception of the wild-type amino acid (P2Y12 codons AAA and AAG isolated from the library and representing the wild-type amino acid at this position were tested) inactivated the receptor completely (Table 1).

Several mutant P2Y12 variants were functionally characterized by the P2Y12 antagonist AR-C66096, for this activation P2Y12 variants with EC_{50} values with ADP < 1.5 μM were selected. In addition, only one mutant receptor variant from the hydrophilic basic, hydrophilic acidic, hydrophilic neutral and hydrophobic amino acid groups was tested. For six of 12 different receptor variants tested, AR-C66096 produced significant shifts in the ADP dose-response curves (Table 1 and Fig. S2).

Quantitative structure–activity relationship

We studied the physiochemical characteristics of E181, R256 and R265 using quantitative structure–activity relationship (QSAR) analysis. For amino acid position 181, the best QSAR model (R^2 = 0.77, Q^2 = 0.55) (Fig. 3A) was obtained using five *z*-scale descriptors from Sandberg *et al.* [28] and adding a charge description to the model. The most important descriptor in this model proved to be the *z*1-scale, representing amino acid hydrophobicity/hydrophilicity (Fig. 3B). The highly positive regression coefficient indicated that hydrophobic amino acids in this position resulted in lower receptor activity. The R256 position was better characterized using the Gottfries *et al.* [29] descriptors system (R^2 = 0.80, Q^2 = 0.60) (Fig. 3C), which revealed a requirement for an amino acid with a smaller rigidity (i.e. without Tyr, Pro or His rings) and higher flexibility (i.e. longer side chains) at this site (Fig. 3D). None of the descriptor combinations gave a highly predictive model for the R265 position based on our data. The best model (R^2 = 0.52, Q^2 = 0.20) was obtained using five *z*-scales (Fig. 3E,F). Positive regression coefficients for the *z*1- and *z*2-scales indicated a preference for an amino acid that was hydrophilic and large at this position. However, analysis of the cross-validation results showed that the model could not find a reason for the threefold difference in activity for receptors containing Asp or Glu, or receptors containing Leu and Ile at this position.

Homology modelling and docking of P2Y12

To investigate the possible role of the tested residues in formation of the ADP-binding pocket of P2Y12, we

Table 1. P2Y12 variants identified from mutagenised libraries and EC_{50} values after ADP stimulation. Underlined, yeast preference codon; wt, wild-type codon; STOP, stop codon; MIX, harvested colonies containing more than one codon variant; NA, no activity observed; NT, not tested. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ significance of differences for wt P2Y12 EC_{50} ± SEM versus mutant P2Y12 EC_{50} ± SEM (*t*-test); ns, non-significant ($P > 0.05$ of differences EC_{50} ± SEM P2Y12 variant activated with ADP versus EC_{50} ± SEM P2Y12 variant activated with ADP + 1 μM AR-C66096 according to *t*-test).

Recombinant P2Y12 variant	Codons	Number of clones	Amino acid	EC_{50} ± SEM (ADP, nM)	EC_{50} ± SEM (ADP + 1 μM AR-C66096, nM)	Dose ratio (EC_{50} AR-C66096 vs. EC_{50} ADP)
wt	GAG	3	–	279 ± 17	749 ± 164	2.68
E181R	CGG	1	Arginine	297 ± 70	5354 ± 1504	18.03
E181K	AAA, AAG	2	Lysine	391 ± 77	NT	–
E181H	CAC	2	Histidine	463 ± 140	NT	–
E181D	GAC, GAT	2	Aspartate	228 ± 15	1291 ± 421	ns
E181A	GCC	1	Alanine	379 ± 37	2194 ± 472	5.79
E181C	TGT	1	Cysteine	529 ± 217	NT	–
E181V	GTA, GTT	2	Valine	627 ± 225	NT	–
E181L	CTA, TTA	3	Leucine	1297 ± 210**	NT	–
E181I	ATC	1	Isoleucine	1188 ± 248*	2197 ± 504	ns
E181F	TTC	1	Phenylalanine	647 ± 262	NT	–
E181N	AAT	1	Aspargine	497 ± 114	NT	–
E181S	AGC, TCT	2	Serine	366 ± 21*	NT	–
E181T	ACC	1	Threonine	733 ± 190	NT	–
E181Y	TAC, TAT	2	Tyrosine	803 ± 282	4006 ± 1262	ns
E181STOP	TAG	1	–	–	–	–
E181MIX	NNN	24	–	–	–	–
R256H	CAC	1	Histidine	13483 ± 1893**	NT	–
R256A	GCG	1	Alanine	9679 ± 75***	NT	–
R256G	GGA, GGC, GGT	9	Glycine	12102 ± 9428	NT	–
R256P	CCA	3	Proline	NA	NT	–
R256I	ATA	4	Isoleucine	1092 ± 19*	5906 ± 1203	5.41
R256N	AAC	5	Asparagine	1704 ± 492*	NT	–
R256Q	CAA, CAG	5	Glutamine	4636 ± 2810	NT	–
R256S	TCT	2	Serine	3214 ± 246***	NT	–
R256Y	TAT	5	Tyrosine	NA	NT	–
R256STOP	TAA, TGA	6	–	–	–	–
R256MIX	NNN	28	–	–	–	–
R265K	AAG	1	Lysine	578 ± 244	511 ± 60	ns
R265E	GAG	1	Glutamate	1701 ± 704	NT	–
R265D	GAC	1	Aspartate	532 ± 245	1906 ± 195	3.58
R265A	GCC	1	Alanine	1189 ± 268*	661 ± 99	ns
R265P	CCT	3	Proline	821 ± 228	NT	–
R265L	CTA, CTG	3	Leucine	1310 ± 223**	NT	–
R265I	ATA	2	Isoleucine	3562 ± 798*	NT	–
R265F	TTC, TTT	2	Phenylalanine	555 ± 128	2350 ± 231	4.23
R265S	TCT	1	Serine	522 ± 141	732 ± 103	ns
R265T	ACA	3	Threonine	607 ± 216	NT	–
R265STOP	TGA	1	–	–	–	–
R265MIX	NNN	31	–	–	–	–
K280R	AGA, CGC, CGG	6	Arginine	NA	NT	–
K280E	GAA, GAG	8	Glutamate	NA	NT	–
K280H	CAT	3	Histidine	NA	NT	–
K280D	GAC	1	Aspartate	NA	NT	–
K280G	GGG, GGT	4	Glycine	NA	NT	–
K280C	TGC	1	Cysteine	NA	NT	–
K280V	GTC, GTG	6	Valine	NA	NT	–
K280P	CCC	1	Proline	NA	NT	–
K280L	CTC, CTG, TTG	11	Leucine	NA	NT	–
K280I	ATA, ATC, ATT	8	Isoleucine	NA	NT	–

Table 1. (Continued).

Recombinant P2Y12 variant	Codons	Number of clones	Amino acid	EC_{50} ± SEM (ADP, nM)	EC_{50} ± SEM (ADP + 1 μM AR-C66096, nM)	Dose ratio (EC_{50} AR-C66096 vs. EC_{50} ADP)
K280F	TTT	5	Phenylalanine	NA	NT	–
K280Q	CAA	1	Glutamine	NA	NT	–
K280S	AGT, TCA, TCT	6	Serine	NA	NT	–
K280Y	ACC, ACG	6	Tyrosine	NA	NT	–
K280STOP	TAA	1	–	–	–	–
K280MIX	NNN	19	–	–	–	–

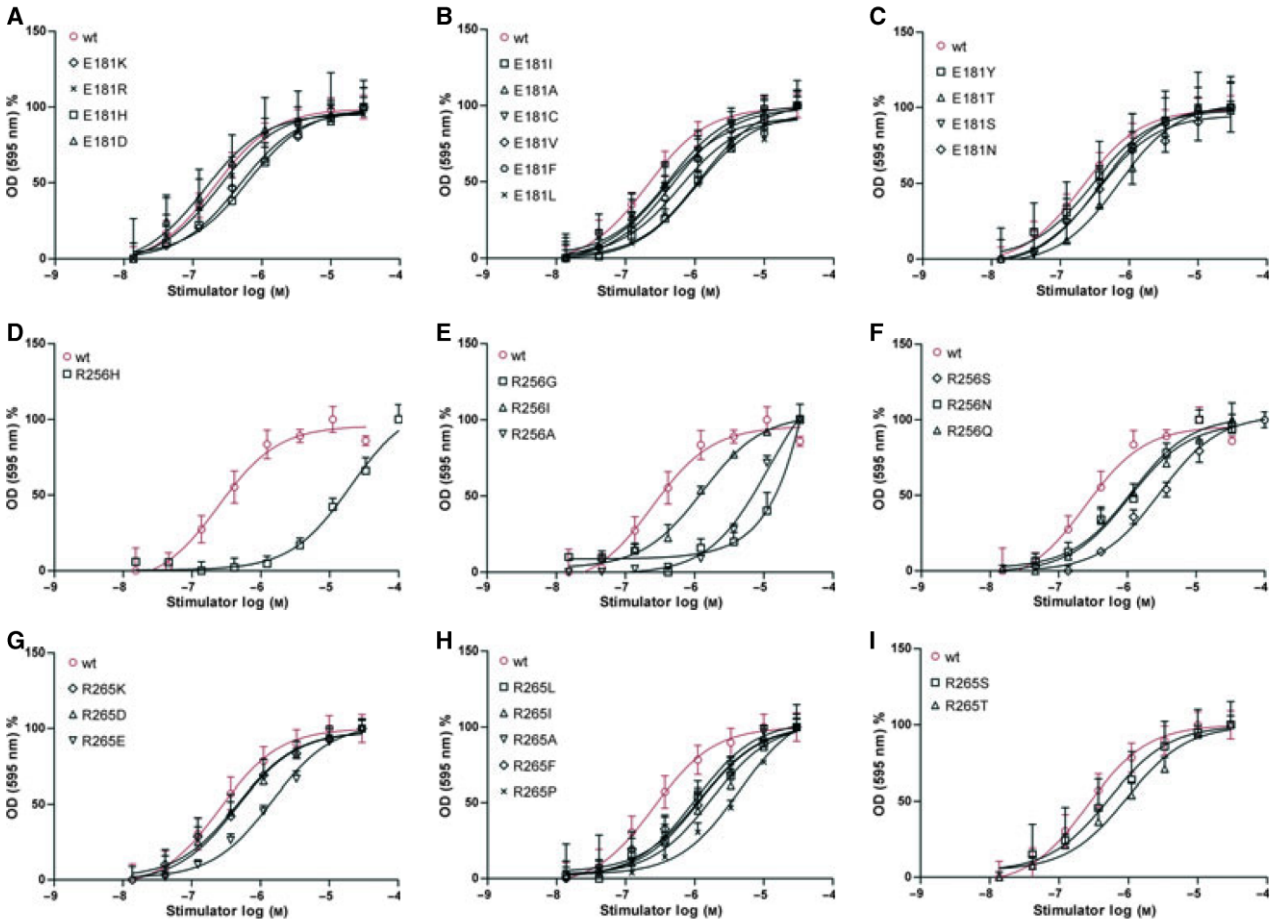

Fig. 2. Activation curves of recombinant P2Y12s. Substitutions with (A,D,G) hydrophilic–charged, (B,E,H) hydrophobic, and (C,F,I) hydrophilic–neutral residues.

generated a homology model of P2Y12 and performed docking studies using ADP as a ligand. The docking model with the lowest free energy is presented in Fig. 4. In our model, the nucleobase group of ADP docked in the cavity between the TM domains. The residues in extracellular loops, E181 and R265, did not appear to be involved in formation of the ADP-binding pocket, whereas amino acids R256 and K280 were located in close proximity of the docked ADP (Fig. 5). Residue K280 was located only 1.5 Å from the ADP phosphate group, suggesting involvement in binding pocket formation.

Discussion

This is one of the first studies to describe mutational analysis of P2Y12 using a yeast system [30] and to discuss advantages of the expression of purinergic receptors

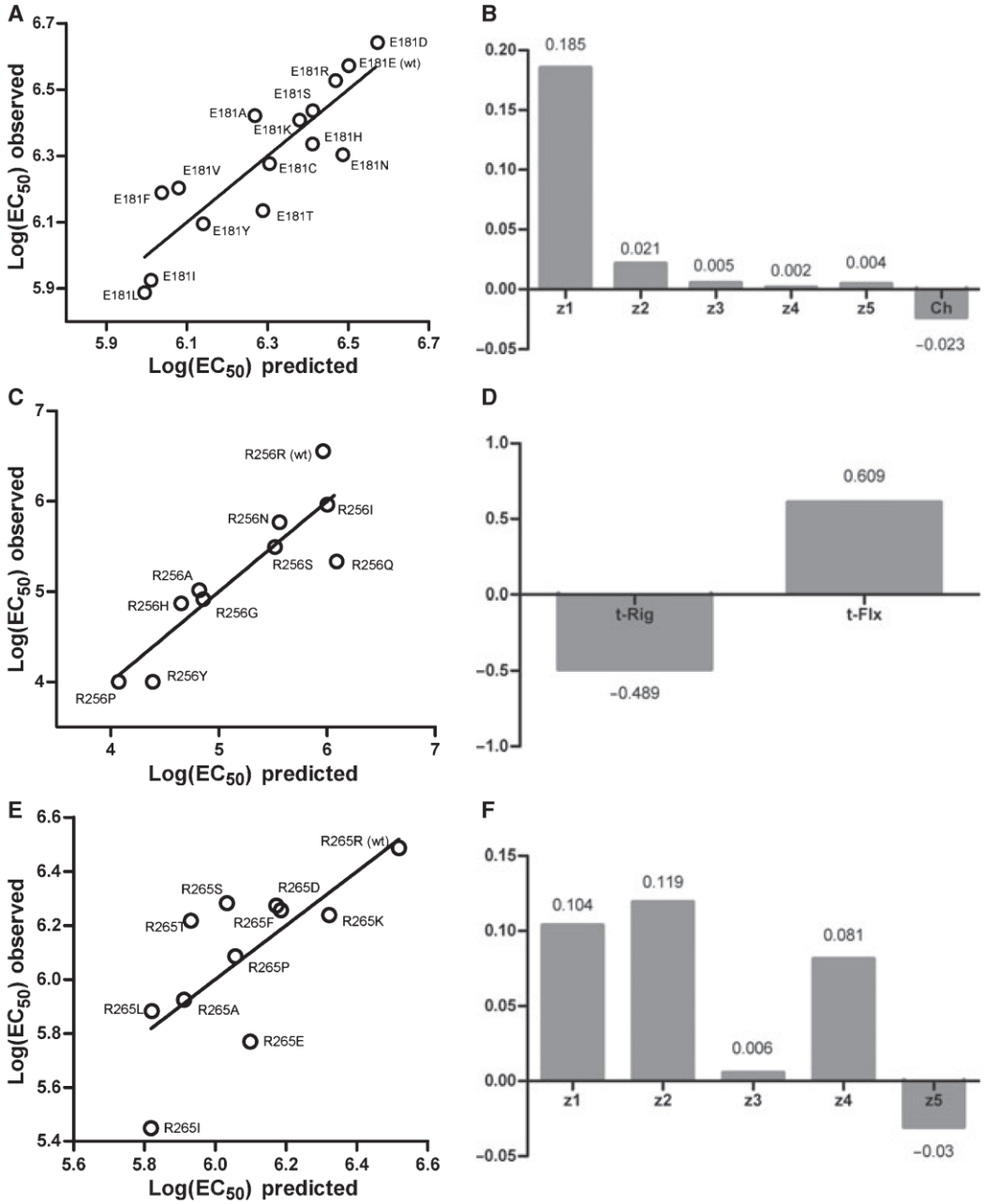

Fig. 3. QSAR models for positions 181, 256 and 265 of P2Y12. Correlation of predicted versus measured activities in models for positions (A) 181, (C) 256 and (E) 265. Orthogonal partial least-square regression coefficients of the used descriptors in models for positions (B) 181, (D) 256 and (F) 265.

in *S. cerevisiae* compared with mammalian cells. We revealed the specific importance of amino acid positions 181, 256, 265 and 280 for functional activation of the receptor and suggested binding pocket characteristics for the natural agonist ADP. We also propose yeast to be a highly sensitive and efficient screening system for P2Y12. EC_{50} values obtained for both ADP (279 ± 17 nM) and the chemically stable ligand 2-methylthio-adenosine-5′-diphosphate (2.66 ± 0.42 nM) are similar to those reported for P2Y12 activation in the mammalian cell systems [31] and in the yeast-based assays [26], indicating that unwanted ADP hydrolysis is not a major issue that may influence the results obtained in our study. This system may be useful for avoiding problems characteristic of mammalian cell cultures. Most of the relevant mutational studies in past have been carried out on other purinergic receptors, and our initial selection of residues was based on knowledge from other P2Y receptors. Recent

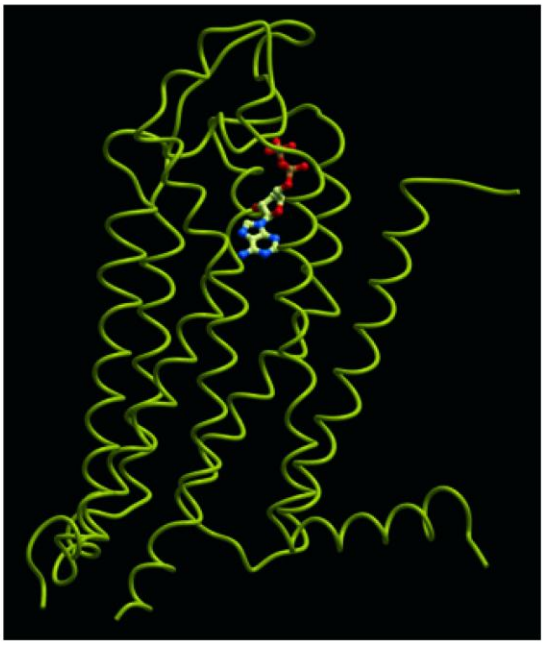

Fig. 4. P2Y12-ADP docking model. ADP docked between TM helices, close to the extracellular side of the receptor cavity.

studies [15,16] have reported mutations of human P2Y12 at positions 256, 265, and 280, allowing us to compare the results obtained in these studies.

A role for EL2 in formation of the ligand-binding pockets in GPCRs has been proposed [32], and evidence supports the involvement of this region in ligand recognition of P2Ys. The amino acid E181 is located in EL2 of P2Y12 and was selected for analysis based on mutagenesis data on E209 and E186 in the EL2 of P2Y1 and P2Y11 that affect the activity of these receptors [20,25]. We extensively explored position 181 of P2Y12 and our data suggested that hydrophobity at this site (when substituted with Leu or Ile) lowered the functional activity of P2Y12, but hydrophilic recombinant receptor variants (Asp, Arg, Lys substitutions) functioned nearly as well as wild-type P2Y12.

Residue R265 is located in the EL3 of P2Y12. Previous studies havce shown that substitution of this position (corresponding to R287 of P2Y1) with hydrophobic (Ala) or hydrophilic–neutral (Gln) amino acids, reduces the functional activity of the receptor by 1000-fold [25]. However, a P2Y12–R265W recombinant receptor showed only a minor change (1.5-fold lower activity) [16] in mammalian cell system. By contrast, our data suggested that even substitution of Arg at the 265 position with the chemically related amino acid Lys led to a twofold decrease in activity.

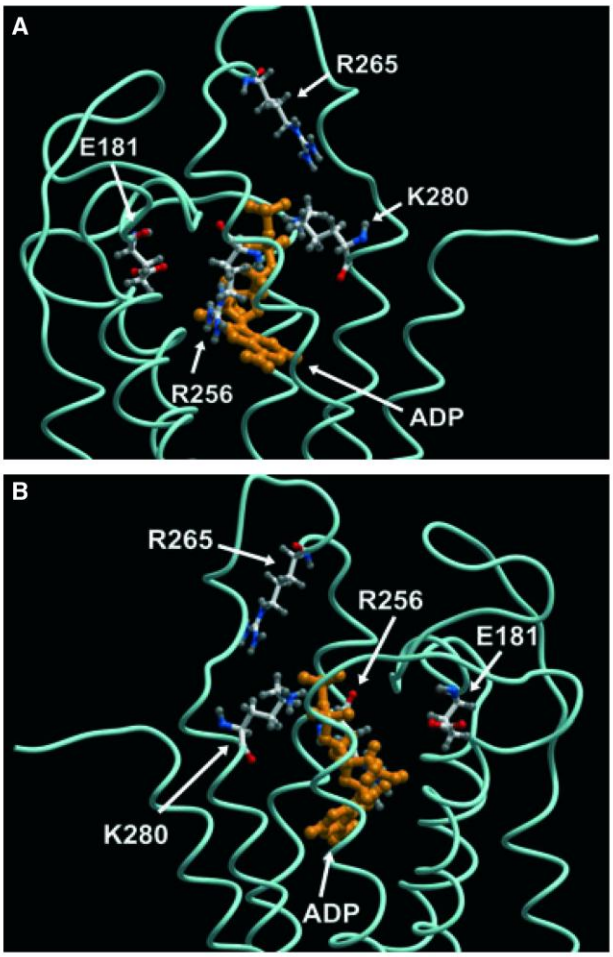

Fig. 5. Detailed view of P2Y12-ADP binding pocket from two viewpoints. (A,B) Four studied amino acids (coloured by atom type) and ADP (amber).

Activation results of the recombinant variants of the R256 library were also in slight disagreement with previous reports. Lys, Ala and Asp recombinant receptor variants have decreased cAMP inhibition after 2-methylthio-adenosine-5′-diphosphate activation [15]. Substitutions of this position with Thr and Gln are shown to have very little effect on P2Y12 activity after ADP stimulation (1.2- and 2.3-fold lower activity, respectively) [16], whereas we observed 17-fold lower activity for the R256Q mutant. It should be noted, however, that these data are not quite comparable with our results because different agonists were used.

Our results might also suggest that expression in the yeast system is more specific for P2Y12 than in most commonly used mammalian cells, supporting assumptions by Schonberg *et al.* [30]. This is somewhat surprising because yeast systems usually have lower potency than mammalian cell cultures [33,34]. However, we observed a P2Y12 activation potential similar to that

described previously [26], and believe that expression of P2Y12 in yeast is highly suitable for this type of study. Similar results have been obtained for P2Y1 receptors where agonists and antagonists were found to be equally potent in the yeast cells and transfected 1321N1 human astrocytoma cells that endogenously do not express P2Y receptors [35]. A number of GPCRs are activated by nucleotide/nucleoside ligands, so other mammalian cell lines are likely to have endogenous background expression of these receptors, leading to inadequate interpretation of activation results. In mammalian cells, P2Y12 might be influenced by conditions such as expression of endogenous P2Y12 ligands or even the presence of a special compensation mechanism when P2Y12 functions are distorted. In conclusion, yeast is a more isolated and straightforward system, with no GPCRs expressed that could lead to possible false-positive results like in mammalian cells.

Interestingly, the QSAR models successfully explained our experimental data on positions E181 and R256. The E181 model proved the necessity of hydrophilic residues at this position for the functional activity of the P2Y12 and this could support the assumption that E181 has important role in ligand binding. However, the question of whether it is a significant component of ligand-binding pocket remains. The R256 model revealed that the most influential characteristic of the amino acid at the 256 position is low rigidity and high flexibility (Fig. 3D). Positively charged amino acids like Arg and Lys are widely thought to be common for all P2Ys in the upper parts of TM helices, and they are believed to form binding pockets for negatively charged nucleotides that are especially attractive to phosphate groups [19,20,25]. Our QSAR results indicate that the R256 position of P2Y12 has specific physical properties that contribute to the spatial organization of the binding pocket but not to the direct interaction of this residue with the ligand.

Docking studies of P2Y12 revealed a possible ADP-binding pocket. We believe that our model is better supported than a previously reported model of P2Y12 [36], because we used data from the A2A adenosine receptor X-ray structure [37] as a template for modelling instead of the bovine rhodopsin receptor. The A2A receptor has a higher amino acid homology to P2Y12 than the bovine rhodopsin receptor and binds a similar class of ligands. Furthermore, the A2A template is in the active state, with bound antagonist, and this more closely mimics the conformation of the receptor activated by ligand *in vivo*.

In our model, the amino acids in the EL of P2Y12 (E181, R265) appear to be far from the docked ligand (Fig. 5). Therefore, the involvement of these residues in binding pocket formation seems implausible. However, if EL has a major role in attracting ligand, EL residues may form a ligand meta-binding site on the outside of the receptor that helps to guide the ligand further into the TM cavity to the final binding pocket. This hypothesis has been proposed previously for P2Y1 [23]. The R256 and K280 residues are close to the docked ADP in our model and might therefore act as the main determinants of receptor–ligand interaction in the binding pocket (Fig. 5). This is supported by our data showing that any substitution at position 280 in P2Y12 leads to nonfunctional P2Y12. Even replacement of the wild-type amino acid Lys with the related amino acid Arg abolished the activity of P2Y12; a minor change in the binding pocket is likely to disarrange it completely (Table 1). Indeed, our docking model demonstrated that K280 is in very close proximity to the second phosphate group on ADP (Fig. 5), supporting this interaction. This could ensure the placement of ADP in the binding pocket within the cavity formed by the TM helices.

Positions 256 and 265 in P2Y12 are reported to be associated with a congenital bleeding phenotype [38]. Interestingly, substitutions of both R256Q and R265W are demonstrated to contribute to a decrease in platelet aggregation, but separately they cause only a minor change in P2Y12 activity [16,38]. We did not attempt to generate the double mutant receptor, but substitution of either position led to a significant decrease in activity (from twofold lower to total abolishment of function). We might have observed this effect because of the higher efficiency and specificity of the yeast expression system, as discussed above, but additional explanations might exist. Studies have demonstrated the role of P2Y1 hetero-oligomerization and its impact on ligand-binding properties [39,40]. If we assume that P2Y12, in mammalian cells as well as *in vivo*, forms dimers with other GPCRs that alter the ligand-binding cavity structure, the double mutant (R256Q and R265W) could decrease ligand binding, whereas a single substitution might not substantially change binding because a more stable structure is formed by hetero-oligomerization. In yeast, however, stabilization by hetero-oligomerization might not take place because of a lack of other endogenously expressed GPCRs.

To estimate the involvement of the analysed amino acids in antagonist binding, we tested the competitive P2Y12 antagonist AR-C66096 on a selected set of mutants. For the majority of substitutions at the E181 and R256 positions, we observed an increase in the dose ratio at 1 μM AR-C66096 (Table 1 and Fig. S2), indicating that none of these amino acids had a strong prevalence for AR-C66096 binding. In the case of

R265K, R265A and R265S substitutions, no significant shifts in ADP response curves were observed, indicating that R265 may be more important for antagonist recognition.

In conclusion, we demonstrated that substitutions at positions 181, 256, 265 and 280 alter the functional activity of P2Y12 in a specific manner, and that these positions are likely to be important for structural and functional modelling of the purinergic receptors. These results can be useful for development of novel antiplatelet agents.

Materials and methods

Cloning of *P2RY12* and construction of mutant libraries

The *P2RY12* gene was cloned from anonymous human DNA, using primers P2RY12-Fw (CCAAAAGCTTATG CAAGCCGTCGACAACCT) and P2RY12-Rs (TA CTCG AGTTACATTGGAGTCTCTTCATTT) (Metabion, Martinsried, Munich, Germany) containing restriction sites *Hin*dIII and *Xho*I (underlined) and were cloned directly into the yeast shuttle vector p426TEF with the URA gene for auxotrophic selection [41]. Consistency of the cloned sequence to the *P2RY12* gene (ID: **64805** in GenBank) was confirmed by sequencing. Mutant P2RY12 libraries were constructed by PCR using a QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, Canada) and oligonucleotides E181rand-Fw (CATGCCAGACTAGACC GAANNNTGATTTAAGGAAAGAGCA), E181rand-Rs (TGCTCTTTCCTTAAATCANNNTTCGGTCTAGTCTGG CATG), K280rand-Fw (TAACCACAGAGTGCTCTC NNNCACATAGAACAGAGTATT), K280rand-Rs (AAT ACTCTGTTCTATGTGNNNGAGAGCACTCTGTGGTTA), R256rand-Fw (TCAGGGTGTAAGGAATNNNGGCAA AATGGAAAGGAAC), R256rand-Rs (GTTCCTTTCCA TTTTGCCNNNATTCCTTACACCCTGA), R265rand-Fw (CAGTGCAGTCAAAGACATCNNNGGTTTGGCTCAG GGTGT) and R265rand-Rs (ACACCCTGAGCCAAA CCNNNGATGTCTTTGACTGCACTG) (Metabion) with randomized trinucleotides (in bold) corresponding to amino acids E181, K280, R256, R265 of P2Y12R and the p426-TEF–P2RY12 construct serving as DNA template. To confirm the heterogeneity of the randomized products, we used direct sequencing with primers P2RY12-Fw and P2RY12-Rs with 3130xl Genetic Analyser (Applied Biosystems, Carlsbad, CA, USA).

Yeast transformation

Yeast cell maintenance, transformation and functional activation of recombinant receptors were as described previously [42]. Briefly, yeast *S. cerevisiae* strain MMY23 with genotype: MATa *his3 leu2 trp1 ura3 can1 gpa1*Δ::*Gs far1*Δ::*ura3 sst2*Δ::*ura3 Fus1*::*FUS1-HIS3 LEU*::*FUS1-lacZ ste2*Δ::*G418*^{R}, was kindly provided by SJ Dowell (Glaxo-SmithKline Medicines Research Centre, Hertfordshire, UK) [43]. This strain expresses the Gi subunit, and has a receptor activation system fused to a specific signalling pathway with reporter genes for β-galactosidase production and histidine synthesis.

Yeast cells were grown overnight in 4 mL YPD medium (20 g·L^{-1} bactotryptone, 10 g·L^{-1} yeast extract; Difco Laboratories, Franklin Lakes, NJ, USA). Transformations with vectors containing the randomized libraries were carried out in 100 mM lithium acetate (Sigma, Deisenhofen, Germany) with 0.2 mg·mL^{-1} salmon sperm DNA (Sigma, St. Louis, MO, USA), with heat shock for 40 min and growth on SC agar (1.92 g·L^{-1} yeast synthetic drop-out medium supplement without uracil, 1.7 g·L^{-1} yeast nitrogen base, 5 g·L^{-1} ammonium sulfate, 2% glucose, 16.67 g·L^{-1} agar; Sigma).

Functional activation of recombinant receptors

Colonies from selective agar plates were harvested in 2 mL liquid SC medium lacking uracil and grown overnight. Functional activation was carried out in 96-well plates (Sarstedt, Nümbrecht, Germany) in 200 μL SC medium without histidine and uracil, containing 2 mM 3-amino-1,2,4-triazole (Sigma), a competitive inhibitor of imidazoleglycerol-phosphate dehydratase for control of background synthesis of histidine, 10× BU salts (70 g·L^{-1} Na_2HPO_4•$7H_2O$, 30 g·L^{-1} NaH_2PO_4 pH 7 adjusted with 2 M NaOH; Reahim, Samara, Russia), 0.1 mg·mL^{-1}chlorophenol red-β-D-galactopyranoside (Roche, Mannheim, Germany) and ligand ADP (Sigma) from 10 nM to 30 μM. Cells were diluted with activation medium to 250 cells·μL^{-1}. For antagonist experiments 1 μM AR-C66096 (Tocris, Bristol, UK) was added. Negative controls for each clone were performed in activation medium without ligand to determine the background activity of the receptor variants. Wt P2Y12 was also activated with 2-methylthio-adenosine-5′-diphosphate (Sigma), to compare with ADP produced response. Cells were grown at room temperature for 24 h or longer. D_{595} was measured on a Victor^3 V reader (Perkin–Elmer, Wellesley, MA, USA).

Analysis of activation data

Data analysis was performed using GRAPHPAD PRISM (GraphPad Software, La Jolla, CA, USA). Data sets were normalized according to highest and lowest values, and transformed using function $X = \log(X)$, and EC_{50} values and standard error of the mean (SEM) were calculated automatically. Significance of differences between wild-type P2Y12 EC_{50} and mutant P2Y12 EC_{50} values as well as significance of antagonist induced EC_{50} shift was estimated by *t*-test.

QSAR

QSAR analysis explored possible physiochemical characteristics of the analysed positions required for P2Y12 functioning. EC_{50} values from functional mutant receptor activation experiments were converted to logarithmic scale. We included inactive recombinant P2Y12 variants in the QSAR models, assigning them an EC_{50} value of 10^5 nM to indicate dramatically decreased receptor activity. Mutated amino acids were described by five *z*-scales, z1 to z5, derived from Sandberg *et al.* [28] *Z*-scales were obtained by principal component analysis of 26 different physicochemical properties of amino acids; they were mutually uncorrelated and essentially represented hydrophobibity/hydrophilicity (z1), steric/bulk properties (z2), polarity (z3) and electronic effects (z4 and z5). Amino acids were also characterized by two descriptors proposed by Gottfries *et al.* [29] (t-Rig, rigidity; t-Flx, flexibility). We added charge as a descriptor (1, positive; −1, negative; 0, not charged). Combinations of these descriptors were explored to obtain a model that best correlated the amino acid properties with the receptor activity from the mutant libraries. Models were created using orthogonal partial least-square regression by SIMCA P-11 software (Umetrics AB, Umea, Sweden). Goodness of fit of the models was estimated by calculating the fraction of the explained activity variation, R^2. Predictive ability was estimated by calculating the fraction of predicted activity variation, Q^2, according to sevenfold cross-validation.

Homology modelling of P2Y12 and docking of ADP

Homology modelling was performed with MOLSOFT ICM-PRO 3.5 software (MolSoft LLC, San Diego, CA, USA) using as a template the 2.6 Å resolution X-ray structure of human A2A adenosine receptor (PDB entry 3EML) [37]. Alignment of sequences was adjusted according to GPCR AlignmentBuilder (http://www.gpcr.org/7tm/align;jsessionid=6f83fb65e2cfa25a405be0c3acdb), which implements a special alignment algorithm for class A (rhodopsin-like) GPCRs. This algorithm is designed to consider conserved residues in TM regions of rhodopsin-like GPCRs, as described previously [44]. A model of P2Y12 was created by the ICM-Homology modelling algorithm as implemented in MolSoft ICM-PRO 3.5. After alignment of the P2Y12 sequence to the template structure and replacement of nonidentical residues, simultaneous global energy optimization was carried out and side chain torsion angles predicted. This procedure was based on biased probability Monte Carlo conformational search and optimization [45,46]. The quality of the generated model was verified by a specialized ICM function that predicted possible backbone deviation between the model and the template [47].

The homology model of P2Y12 was used for ADP docking, using the ICM-Docking algorithm in MolSoft ICM-PRO 3.5. First, a binding pocket was identified and receptor energy maps generated with grid cell size 0.5 Å. Flexible ligand docking was then carried out from multiple conformations and locations. Finally, refinement of a P2Y12 structure was made for all docked ADP conformations to adjust the receptor conformation to the ligand. The docking model with the lowest free energy value was considered the most likely to correspond to *in vivo* binding.

Acknowledgements

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Supporting information

The following supplementary material is available:
Fig. S1. Activation curves of wt P2Y12 after stimulation with ADP and 2MeSADP.
Fig. S2. Activation curves of recombinant P2Y12s with ADP and ADP + 1 μM AR-C66096. (A) wt P2Y12R, (B) E181R, (C) E181D, (D) E181A, (E) E181I, (F) E181Y, (G) R256I, (H) R265K, (I) R265D, (J) R265A, (K) R265F, (L) R265S.

This supplementary material can be found in the online version of this article.

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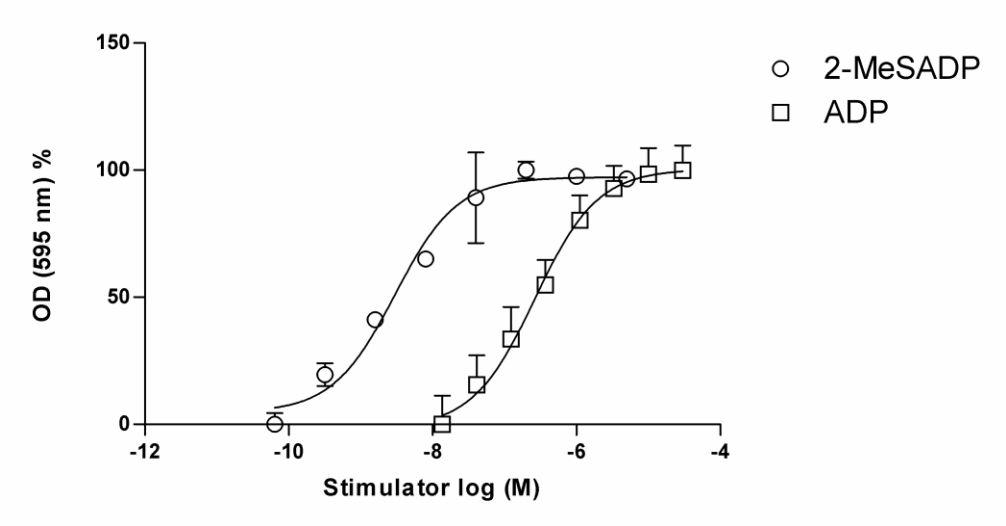

Figure S1. Activation curves of wt P2Y12R after stimulation with ADP and 2MeSADP.

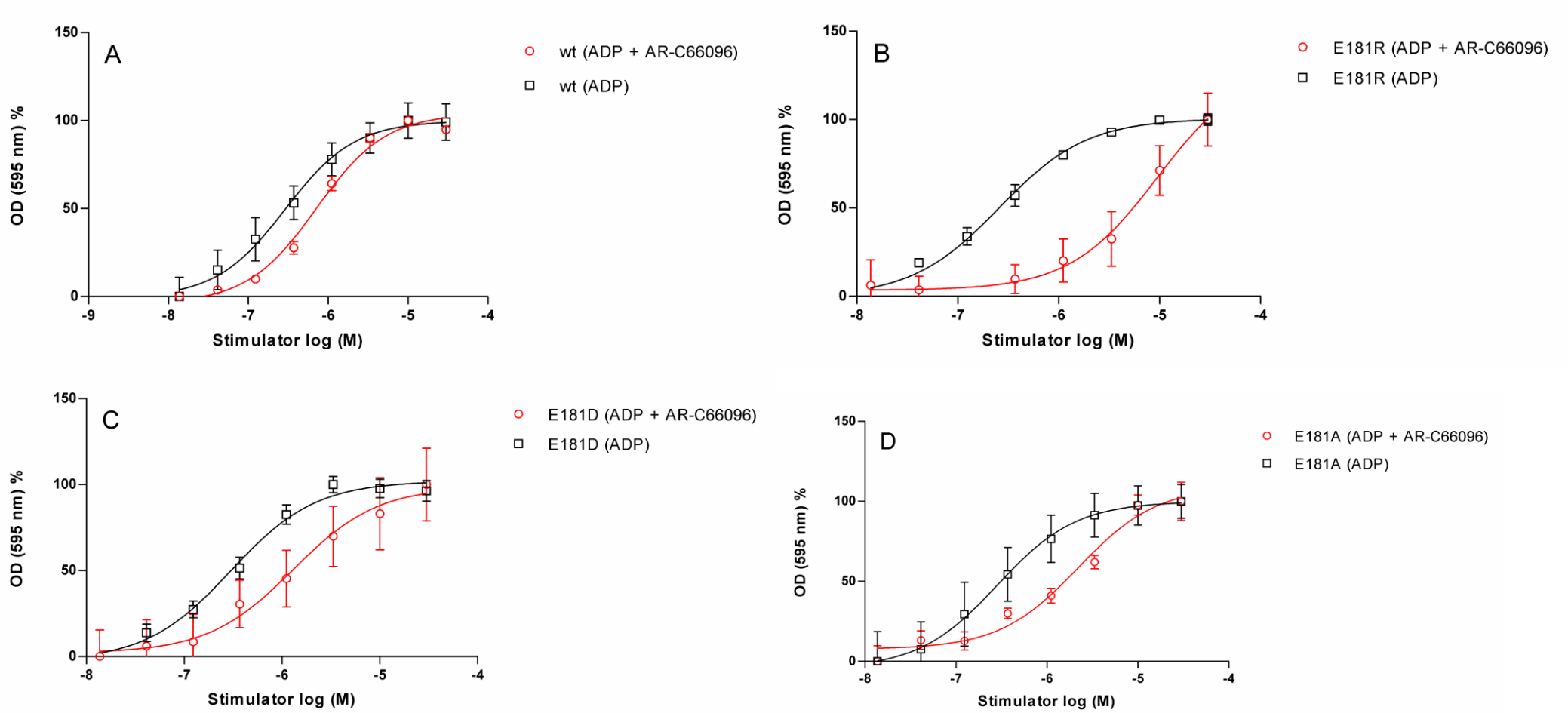

Figure S2. Activation curves of recombinant P2Y12Rs with ADP and ADP + 1µM AR-C66096. (A) wt P2Y12R, (B) E181R, (C) E181D, (D) E181A.

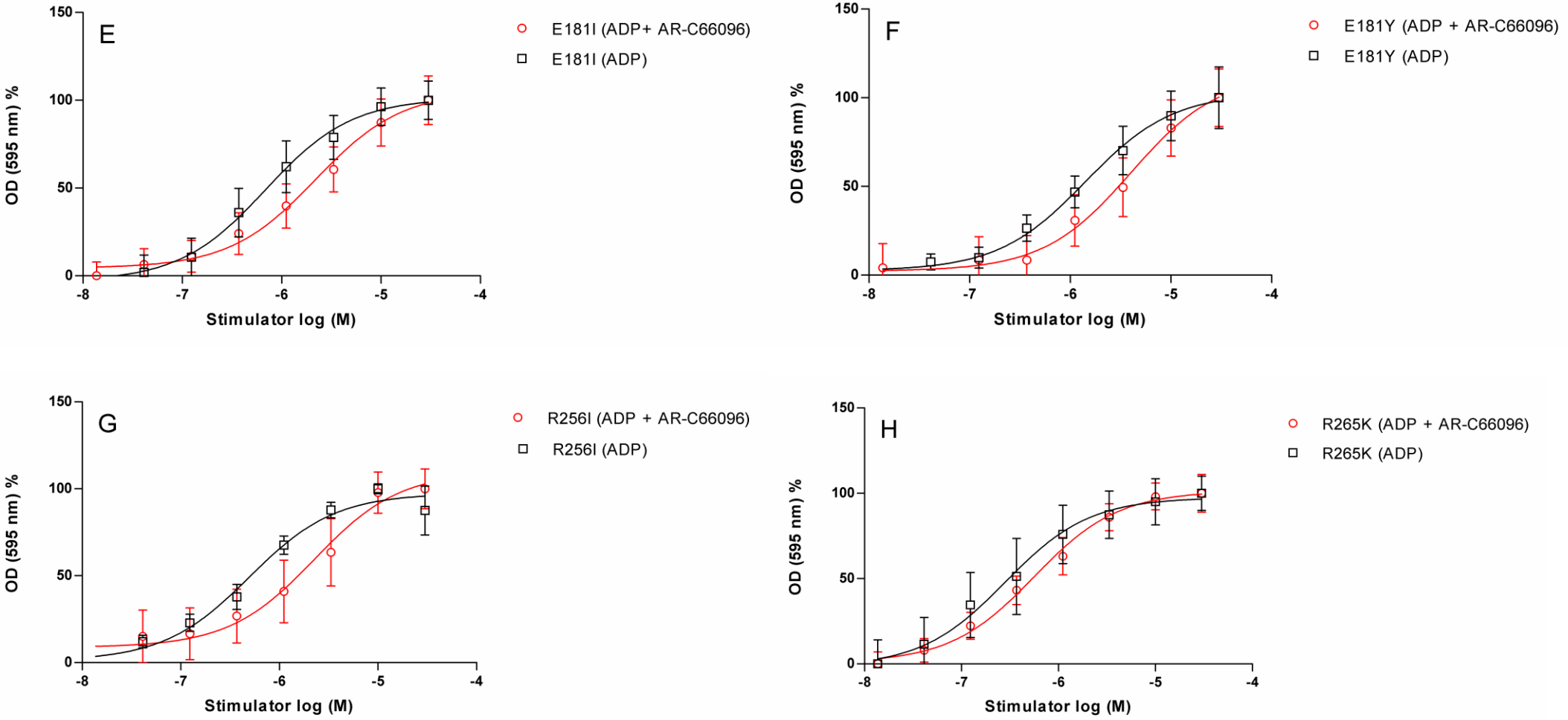

Figure S2. Activation curves of recombinant P2Y12Rs with ADP and ADP + 1μM AR-C66096. (E) E181I, (F) E181Y, (G) R256I, (H) R265K.

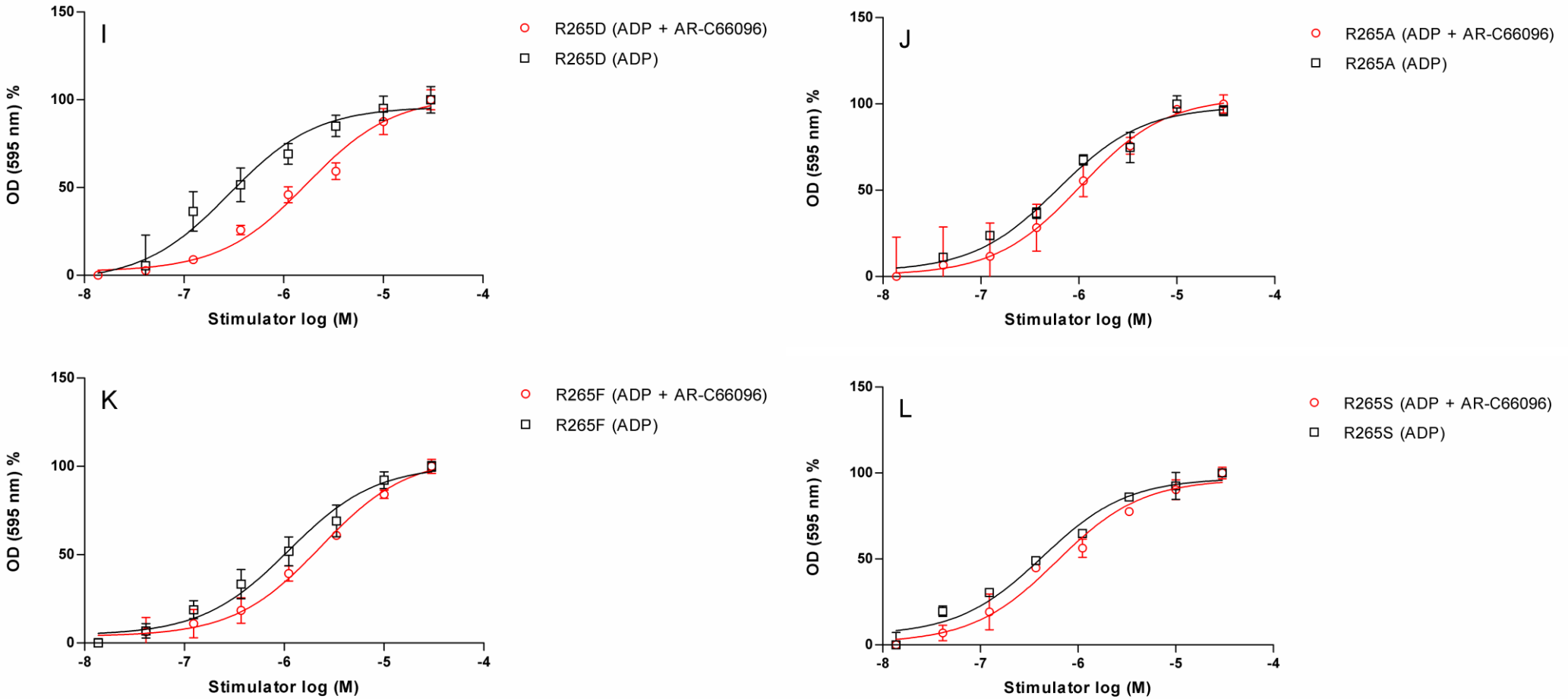

Figure S2. Activation curves of recombinant P2Y12Rs with ADP and ADP + 1μM AR-C66096. (I) R265D, (J) R265A, (K) R265F, (L) R265S.

3.4 Analysis of common and rare *MC4R* variants and their role in extreme form of obesity

Analysis of common and rare *MC4R* variants and their role in extreme form of obesity

Running head: *Analysis of MC4R in relation to obesity*

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Abstract

Melanocortin 4 receptor (MC4R) is an important regulator of food intake and number of studies report genetic variations influencing the risk of obesity. Here we explored the role of common genetic variation from *MC4R* locus comparing with SNPs from gene *FTO* locus, as well as the frequency and functionality of rare *MC4R* mutations in cohort of 380 severely obese individuals (BMI > 39 kg/m^2) and 380 lean subjects from the Genome Database of Latvian Population (LGDB).

We found correlation for two SNPs - rs11642015 and rs62048402 in the fat mass and obesity-associated protein (FTO) with obesity but no association was detected for rs17782313 located in the *MC4R* locus in these severely obese individuals. We sequenced the whole gene *MC4R* coding region in all study subjects and found four previously known heterozygous nonsynonymous substitutions V103I, I121T, S127L, I251L and one novel mutation V166I. Expression in mammalian cells showed that the S127L, V166I and double V103I/S127L mutant receptors had significantly decreased quantity at the cell surface compared to the wild type MC4R. Despite low abundance in the plasma membrane, the novel V166I variant demonstrated higher cAMP response upon αMSH activation than the wild type receptor, while the level of AGRP inhibition was lower, implying that V166I cause hyperactive satiety signalling. Overall, this study suggest that S127L may be the most frequent functional *MC4R* mutation leading to the severe obesity in general population and provides new insight into the functionality of population based variants of the *MC4R*.

Introduction

The melanocortin 4 receptor (MC4R) is a plasma membrane G protein-coupled receptor. It is mainly expressed in endocrine regions of the brain ventromedial, dorsomedial, paraventricular and arcuate hypothalamic nuclei, where it controls feeding behaviour (Cone, 2005; Mountjoy *et al.*, 1994). Furthermore, MC4R knockout mice exhibit obese, hyperphagic and hyperinsulinemic phenotype (Huszar *et al.*, 1997) and MC4R antagonist and agonist are very potent in animal feeding studies (Kask *et al.*, 1998). There are number of studies that link variants of the *MC4R* with human obesity and feeding behaviour related phenotypes. It is also known that several different variants within the coding region of the *MC4R* can affect the functionality of the MC4R, in some cases even causing monogenic forms of childhood obesity (Biebermann *et al.*, 2003). A detailed view of rare alterations in the *MC4R* is presented in Figure 1.

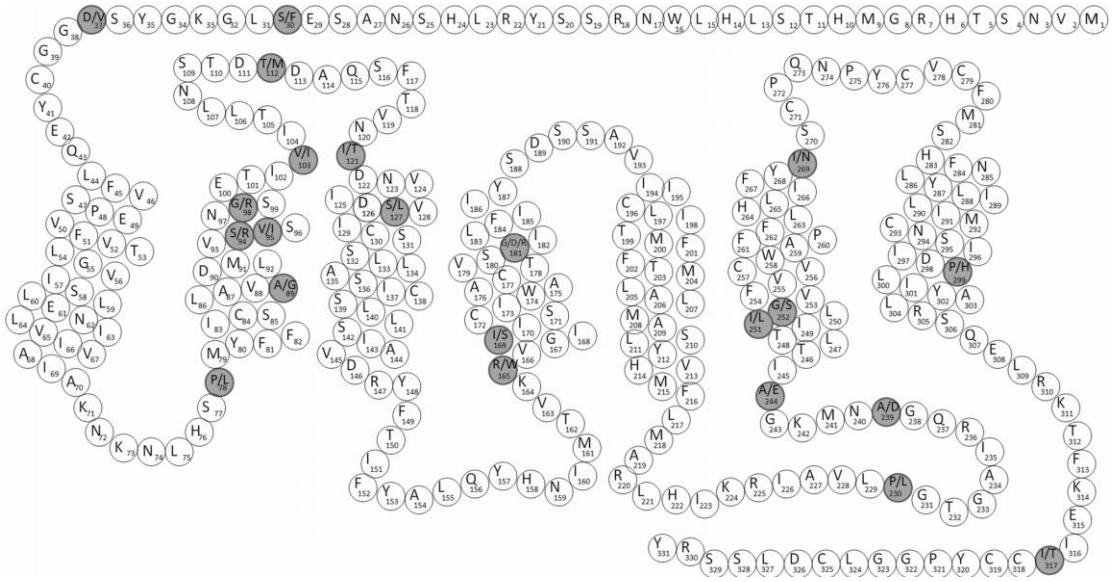

Figure 1. Schematic diagram of the melanocortin 4 receptor. Highlighted in gray are nonsynonymous coding amino acid substitutions described in literature, also frameshift coding deletions in positions L322, I251, L211 and deletion in position Y35 leading to stop codon has been reported (data from Ensemble Genome Browser, www.ensembl.org)

Most of these substitutions are functionally relevant and characterization of nonsynonymous *MC4R* variants has been performed in various studies. These have

demonstrated involvement of D37V, P78L R165W, P299H, I317T in receptor protein trafficking to cell membrane, while P78L, S94R, V95I, I121T, S127L, G181D, P230L, A244E substitutions have shown the impact on signal transduction mechanisms, but T112M and I169S *MC4R* variants had equal trafficking and transduction efficacy as the wild-type receptor (Calton *et al.*, 2009; Fan *et al.*, 2009; Hinney *et al.*, 2003; Lubrano-Berthelier *et al.*, 2003; Valli-Jaakola *et al.*, 2004; Xiang *et al.*, 2006). Functional studies of two of the most common substitutions V103I and I251L have not demonstrated significant changes in functionality compared to the wild-type *MC4R*, but genetic association studies indicate that both variant could have protective effect against obesity related conditions (Mirshahi *et al.*; Stutzmann *et al.*, 2007; Wang *et al.*, 2010; Young *et al.*, 2007).

Common genetic polymorphic variants in non-coding part of the *MC4R* locus have also been implicated in obesity development. Thorough meta-analysis of several genome wide association studies (GWAS) (Consortium, 2007) performed in 2008 showed association of the *MC4R* region with BMI (Loos *et al.*, 2008). Initial scan of over 16,000 people pointed out strong significant association of rs17782313, located upstream of the *MC4R* with increased BMI. These finding have been replicated in several populations, covering more than 44,000 people of European descent (Loos *et al.*, 2008). The association of rs17782313 has been replicated by different research groups to various obesity related phenotypic traits such as - BMI, waist circumference, body weight, hypertension, some characteristics of body fat distribution and other phenotypes in different cohorts (Hardy *et al.*, 2010; Haupt *et al.*, 2009; Timpson *et al.*, 2009; Zobel *et al.*, 2009). Today the genes that are most studied in relations to human obesity are probably the *MC4R* and the fat mass and obesity associated protein *FTO* (Frayling *et al.*, 2007).

In this study we evaluated both the common genetic variant rs17782313 and nonsynonymous substitutions identified by the sequencing the coding part of *MC4R* in relation to severe obesity in Latvian population in comparison with three SNPs in gene *FTO* locus. Moreover, we generated five recombinant constructs of the MC4R variants: V103I, S127L, V166I, I251L and double mutant containing both V103I and S127L, functionally expressed these mutants and estimated receptor quantity in cell surface and signal transduction efficacy.

Methods and Procedures

Study group

The STrengthening the REporting of Genetic Associations (STREGA) guidelines (Little *et al.*, 2009) were used for the design of the study group and statistical analysis. Groups of cases and controls were selected from the Latvian Genome Data Base (LGDB), a government funded biobank (shortly described in (Ignatovica *et al.*, 2011)).

The study group for this analysis was selected from all LGDB participants recruited from 2003 till May of 2011 (n=16503). We excluded participants: with missing important phenotypic data, with body mass associated conditions like cancer (ICD: from C00 to C97), hyperthyroidism related diseases (ICD: from E00 to E07), other endocrine diseases (ICD: from E20 to E27) and professional athletes. For the obesity study group, we selected 380 individuals from the extreme end of the BMI scale (to match four 96 well plate format, including 95 participants and one negative control in each plate). For the control group, we randomly selected 380 individuals from normal weight group (BMI 18.5 – 24.9) (total n=2129) maintaining the same sex proportion and average age as in the case group.

Genotyping by RT-PCR

Stock DNA samples acquired from the LGDB were diluted into 95 wells of 96-well PCR plates (28 ng per well) using the Freedom Evo robotic workstation (Tecan, Männedorf, Switzerland) with disposable filter tips, one position in the plate was filled with Millipore (Millipore, Bedford, USA) H_2O for negative control. Genotyping was performed using an Applied Biosystems TaqMan SNP Genotyping Assay (Applied Biosystems, USA) as previously described (Peculis *et al.*, 2011). SNP genotyping assays used for rs17782313 ID:C_32667060_10, for rs11642015 ID:C_2031268_20, for rs9939609 ID:C_30090620_10 and for rs62048402 custom made probe was used (Applied Biosystems, USA). Genotypes were assigned using AutoCaller 1.1 (Applied Biosystems, USA) software and manually verified.

Statistical analysis

Statistical analysis was carried out as implemented in Plink 1.06 software (Purcell *et al.*, 2007) (http://pngu.mgh.harvard.edu/purcell/plink/). The deviation from Hardy-Weinberg equilibrium was tested using Plink. The χ^2 test was performed to compare case-control groups. The additive model of inheritance was used in logistic regression for each analysed polymorphism adjusting for sex and age. Power calculations were done in Quanto v1.2.3 (Gauderman *et al.*, 2006) (http://hydra.usc.edu/gxe/). Population risk for obesity was set at 0.2 and α was set at 0.05. The allele frequencies were used as previously reported – for rs17782313 - 0.25, for rs9939609 - 0.46 and for both rs11642015 and rs62048402 - 0.41 (Frayling *et al.*, 2007; Loos *et al.*, 2008; Sallman Almen *et al.*, 2012). An additive mode of inheritance was used for power estimation.

Sequencing of *MC4R*

DNA samples were prepared as described above. PCR primers for amplification of genomic DNA and sequencing of *MC4R* were designed by modified Primer3 program (http://primer3.sourceforge.net/) and synthesized by Metabion (Martinsried, Germany). Two sets of primers were used to amplify entire coding region of the

MC4R: pcrFw1 and MC4RpcrRs1, MC4RpcrFw2 and MC4RpcrRs2 (all primer sequences are presented in Supplementary Material 1). Amplification was carried out by standard polymerase chain reaction (PCR), using a reaction mix containing 1mM DB buffer, 2.5mM MgCl2, 0.5 units (U) HotFire polymerase, 0.2mM dNTP mix (all from SolisBioDyne, Tartu, Estonia), 0.3mM each primer, and 28ng template DNA. PCR was carried out on a Veriti96 ThermalCycler (Applied Biosystems, USA) using the following conditions: denaturation at 95^0C for 5 min, 40 cycles of amplification at 95^0C for 30 sec, 55^0C for 30 sec, and 72^0C for 1 min, and final extension at 72^0C for 5 min. Products were confirmed by visualization on agarose gel after electrophoresis. Dephosphorylation and degradation of unused dNTP and primers in the reactions was carried out by addition of shrimp alkaline phosphatase and exonuclease I (Fermentas, Lithuania) according to the manufacturer's protocol. For sequencing two sets of primers were used - MC4RseqFw1 and MC4RseqRs1, MC4RseqFw2 and MC4RseqRs2. Sequencing of PCR products was performed using reaction mix containing 5µl Millipore H_2O, 2µl sequencing buffer, 0.5µl BigDye (both from Applied Biosystems, USA), 0.5µl primer using the following conditions denaturation at 95^0C for 5 min, 25 cycles of 95^0C for 15 sec, 50^0C for 5 sec, 60^0C for 4 min. Purification of sequencing products was performed with Sephadex following the manufacturer's protocol (Sigma, Germany). Sequencing analysis was performed on 3130xl Genetic Analyzer (Applied Biosystems, USA). All chromatograms were manually inspected using ContigExpress software as implemented in VectorNTI (Invitrogen, USA).

MC4R mutant expression microscopy and activation in BHK cells and statistical analysis

MC4R was amplified from anonymous human genomic DNA using MC4R-Fw and MC4R-Rs primers (Supplementary Material 1) containing *HindIII* and *XhoI* restriction sites and cloned directly into mammalian cell vector pCEP-GFP-C, modified from pCEP4 (Invitrogen, USA) with insertion of the enhanced green fluorescent protein gene at the 3' end of the multiple cloning site. Consistency of the cloned sequence to the gene *MC4R* (ID: 4160 in GenBank) was confirmed by sequencing. Mutant *MC4R* were constructed by PCR using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, Canada) and oligonucleotide primers (presented in Supplementary Material 1) following manufacturers' protocol. Nucleotide substitutions in the gene *MC4R* products were confirmed by sequencing.

For cAMP and microscopy experiments baby hamster kidney (BHK) cells were grown in DMEM medium supplemented with 10% fetal calf serum (both purchased from Sigma, Germany) to 70% monolayer density. Vector transfection was carried out using TurboFect^{TM} *in-vitro* Transfection Reagent (ThermoScientific, USA) following the manufacturer's protocol. For microscopy, cells were grown for 24 h after transfection in 12-well plate containing microscopy slip and labelled for 2 min in AlexaFluor 633-labeled wheat germ agglutinin (AF-WGA) (Invitrogen, USA) and fixed with formaldehyde (Sigma, Germany) for 10 min. Microscopy slips were then placed on microscopy slides and inspected with confocal fluorescent microscope Leica DM 600B (Leica, Germany). At least three independent transfections were carried out for each receptor mutant for microscopy analysis. Statistical analysis of confocal microscopy was carried out as previously described (Fridmanis *et al.*, 2010).

For cAMP response assays cells were grown for 36-48 h after transfection and functionally tested with αMSH (melanocytes stimulating hormone) (PolyPeptide

group, France) and AGRP (83-132) (Phoenix Pharmaceuticals inc., USA). Intracellular cAMP labelling was carried out with LANCE cAMP 384 kit (PerkinElmer, USA) following the manufacturer's protocol and measured on plate reader VictorV3 (PerkinElmer, USA). The cAMP concentrations were quantified by comparison to a standard curve of the control concentrations of cAMP provided in a kit. Analysis of αMSH and AGRP activation data was done using GraphPad Prism software as previously described (Ignatovica *et al.*, 2012).

Results

Phenotypic characteristics of the study groups are given in Table 1. Both groups were well matched for sex proportion and age. Apart from BMI, significant differences between cases and controls existed with respect to presence of hypertension, hyperlipidemia and type 1 and 2 diabetes, while presence of *angina pectoris*, myocardial infarction and other types of diabetes did not differ significantly between the study groups.

Table 1 **Characteristics of the study group**

Characteristic	Cases (380)	Controls (380)	*p*-value
Female, n (%)	265 (70%)	265 (70%)	1
Male, n (%)	115 (30%)	115 (30%)	1
Mean age, (±SD) years	57 ± 11.7	57 ± 12.3	1
Mean BMI, (±SD) kg/m^2	44.5 ± 5.0	22.9 ± 1.6	<0.0001
Hypertension, n (%)	253 (67%)	129 (34%)	<0.0001
Angina Pectoris, n (%)	89 (23%)	74 (19%)	0.2060
Myocardial infarction, n (%)	81 (21%)	86 (23%)	0.5633
Type 2 diabetes, n (%)	204 (54%)	36 (9%)	<0.0001
Type 1 diabetes, n (%)	4 (1%)	19 (5%)	0.0025
Other type of diabetes, n (%)	1 (0.3%)	5 (1.3%)	0.2542

BMI - body mass index, SD - standard deviation

All samples (n=760) were genotyped for rs17782313, rs9939609, rs11642015 and rs62048402 polymorphisms using RT-PCR. The genotyping success, minor allele frequencies and deviation from Hardy-Weinberg equilibrium are shown in Supplementary Material 2. All SNPs were in Hardy-Weinberg equilibrium and genotyping success was higher than 99.3 %.

According to power calculations, our sample size provided 80% power (at α = 0.05) to detect odds ratio (OR) above 1.4 for MC4R SNP rs17782313 and 1.35 for SNPs in the gene *FTO* (Supplementary Material 3).

Table 2 shows the results of association analysis of four studied SNPs and obesity (adjusted for sex and age). rs11642015 and rs62048402 were in complete

linkage disequilibrium (LD and statistical analysis of these SNPs is presented together (Table 2)). rs17782313 from *MC4R* locus was not associated with obesity. We, however, observed significantly increased frequency of A and C alleles for the gene *FTO* SNPs rs11642015 and rs62048402, respectively, in the group of severely obese patients ($p_{perm} = 0.042$).

Table 2 **Association of obesity with rs17782313, rs9939609, rs11642015 and rs62048402**

SNP (Gene)	Genotypes	Genotype distribution		OR [95% CI][b]	*P* – value[b]
		Cases	Controls		
rs11642015[a] rs62048402[a] (*FTO*)	CC/CT/TT GG/GA/AA	107/184/89	80/192/108	1.272 [1.04-1.56]	0.0192[c]
rs9939609 (*FTO*)	TT/TA/AA	102/184/94	79/194/107	1.208 [0.99-1.48]	0.0658
rs17782313 (*MC4R*)	TT/TC/CC	17/119/241	17/109/253	1.091 [0.85-1.40]	0.4935

[a] rs11642015 and rs62048402 were in 100% LD, [b] *P*-value and OR was estimated using logistic regression and adjusted for age and sex, [c] 100,000 permutations $p_{perm} = 0.042$

We searched for presence of any sequence alterations within the *MC4R* gene in obesity and control groups by sequencing of the entire coding region. We found five different nonsynonymous single nucleotide substitutions in heterozygous state. Three previously known variants V103I, I251L and S127L and one novel substitution V166I (Supplementary Material 4) were found in the group of obese patients. Three previously described variants V103I, I251L and I121T were found in the controls. The occurrence of these variants in cases and controls are shown in Table 3, except for I121T that was only found in one individual. Two people had *MC4R* variant with double substitutions V103I and S127L that according to haplotype analysis are localized on the same copy of *MC4R*.

To evaluate the possible role of these mutations in the obesity group on functionality of the receptor, we generated five recombinant constructs of the MC4R:

V103I, S127L, V166I, I251L and double mutant containing both V103I and S127L. We estimated receptor quantity in cell surface and signal transduction efficacy for all constructs. Confocal microscopy followed by statistical analysis revealed that mutant MC4R containing S127L, V166I and double mutation V103I and S127L had significantly decreased cell surface expression when compared to wt MC4R (Figure 2, Figure 3). The melanocortin 2 receptor (MC2R) that reported to be detained in endoplasmic reticulum when expressed in mammalian cells (Webb *et al.*, 2009) was used as control that corresponds to no trafficking to the cell membrane (Figure 2, Figure 3).

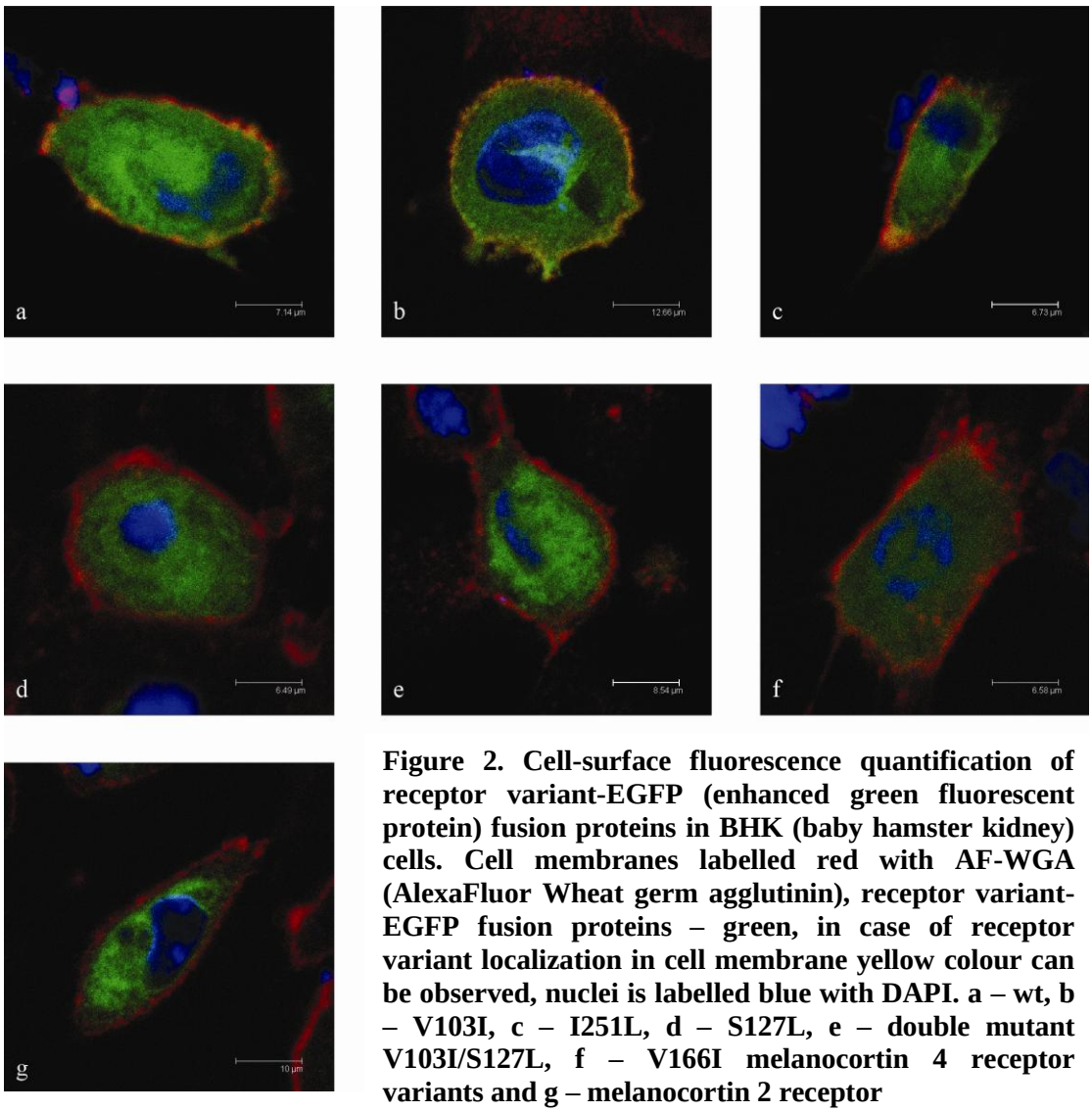

Figure 2. Cell-surface fluorescence quantification of receptor variant-EGFP (enhanced green fluorescent protein) fusion proteins in BHK (baby hamster kidney) cells. Cell membranes labelled red with AF-WGA (AlexaFluor Wheat germ agglutinin), receptor variant-EGFP fusion proteins – green, in case of receptor variant localization in cell membrane yellow colour can be observed, nuclei is labelled blue with DAPI. a – wt, b – V103I, c – I251L, d – S127L, e – double mutant V103I/S127L, f – V166I melanocortin 4 receptor variants and g – melanocortin 2 receptor

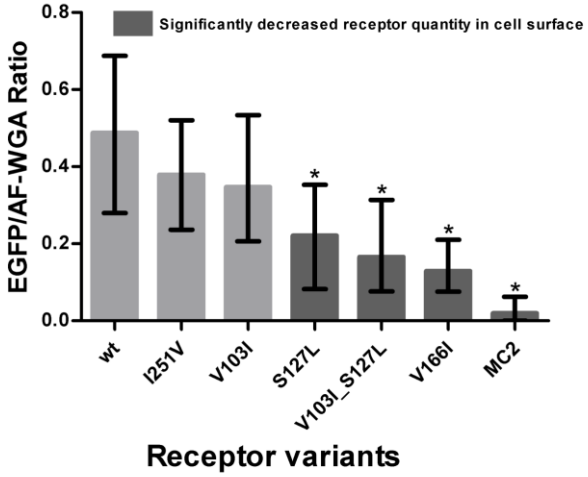

Figure 3. Medians with interquartile ranges of EGFP/AF-WGA (enhanced green fluorescent protein/AlexaFluor Wheat germ agglutinin) ratio in the cell membrane of the melanocortin 4 receptor variants and melanocortin 2 receptor. Columns with dark grey colouring and asterisk represent receptor variants that have significantly decreased transport to cell membrane (estimated using Kruskal-Wallis test)

Pharmacological analysis of recombinant mutant MC4R obtained from the cAMP assay are shown in Table 3 and Figures 4 and 5. The I251L and V103I variants demonstrated EC_{50} values similar to the wt MC4R upon activation with αMSH. Interestingly, the V166I mutant had significantly lower EC_{50} value and higher increase in cAMP accumulation compared to wt MC4R after αMSH activation (Table 3, Figure 4. a, c). Due to low affinity of S127L and double mutant V103I/S127L to αMSH, we were unable to calculate EC_{50} (Table 3, Figure 4. b, d). Interestingly, this double V103I/S127L mutant displayed some increase in cAMP accumulation at highest αMSH concentration compared to S127L (Figure 4. d).

Table 3 **Effects of αMSH stimulation on MC4R mutants**

Mutant MC4R variant	Number in controls[a]	Number in cases[a]	EC_{50} ± SEM (αMSH, nM)	EC_{50} ± SEM (αMSH + 100μM AGRP, nM)	Dose Ratio (EC_{50} AGRP vs. EC_{50} αMSH)
wt	-	-	326 ± 61	4249 ± 818	13.03
I251L	2	2	196 ± 27	2104 ± 93	10.73
V103I	12	8	210 ± 55	20140 ± 7801	95.90
S127L	0	1	nd	nd	-
V103I/S127L	0	2	nd	nd	-
V166I	0	1	59 ± 3[b]	238 ± 121[b]	4.03

nd - not determinable, because cAMP response curves formed no plateau, [a] all mutations were in heterozygous state, [b] $P < 0.05$ significance of difference for wt MC4R EC_{50} ± SEM vs. mutant EC_{50} ± SEM (*t*-test)

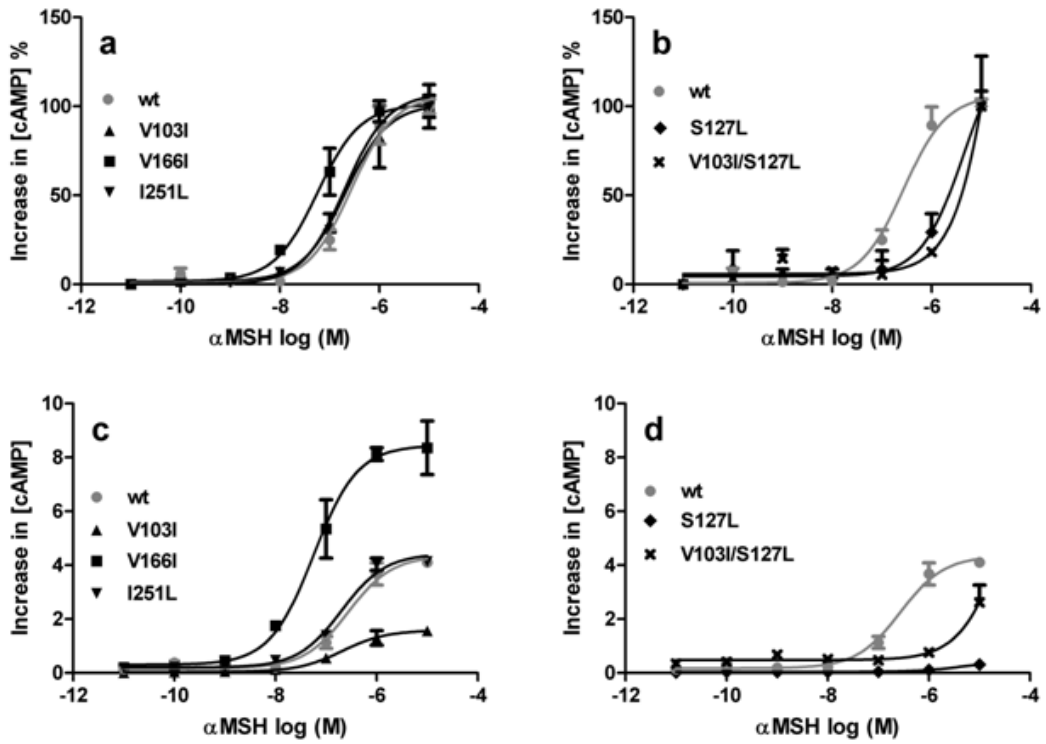

Figure 4. Cyclic adenosine monophosphate (cAMP) response curves of melanocortin 4 receptor mutants in BHK (baby hamster kidney) cells. a, b – cAMP concentration is expressed as percentage with the basal level set to 0% and the highest level in each data set to 100%, c, d – relative increase in cAMP concentration

To evaluate the possible impact of these alterations on natural antagonist AGRP binding to the receptor, we performed activation of all mutants with addition of 100 μM AGRP (Table 3, Figure 5). Dose ratio for the I251L receptor was comparable with that of wt MC4R. The V103I mutant demonstrated highly increased EC_{50} value compared to wt MC4R upon AGRP inhibition (Table 3) while the V166I variant on the other hand showed smaller receptor affinity curve shift in presence of AGRP comparing to other tested MC4R variants (Table 3, Figure 5).

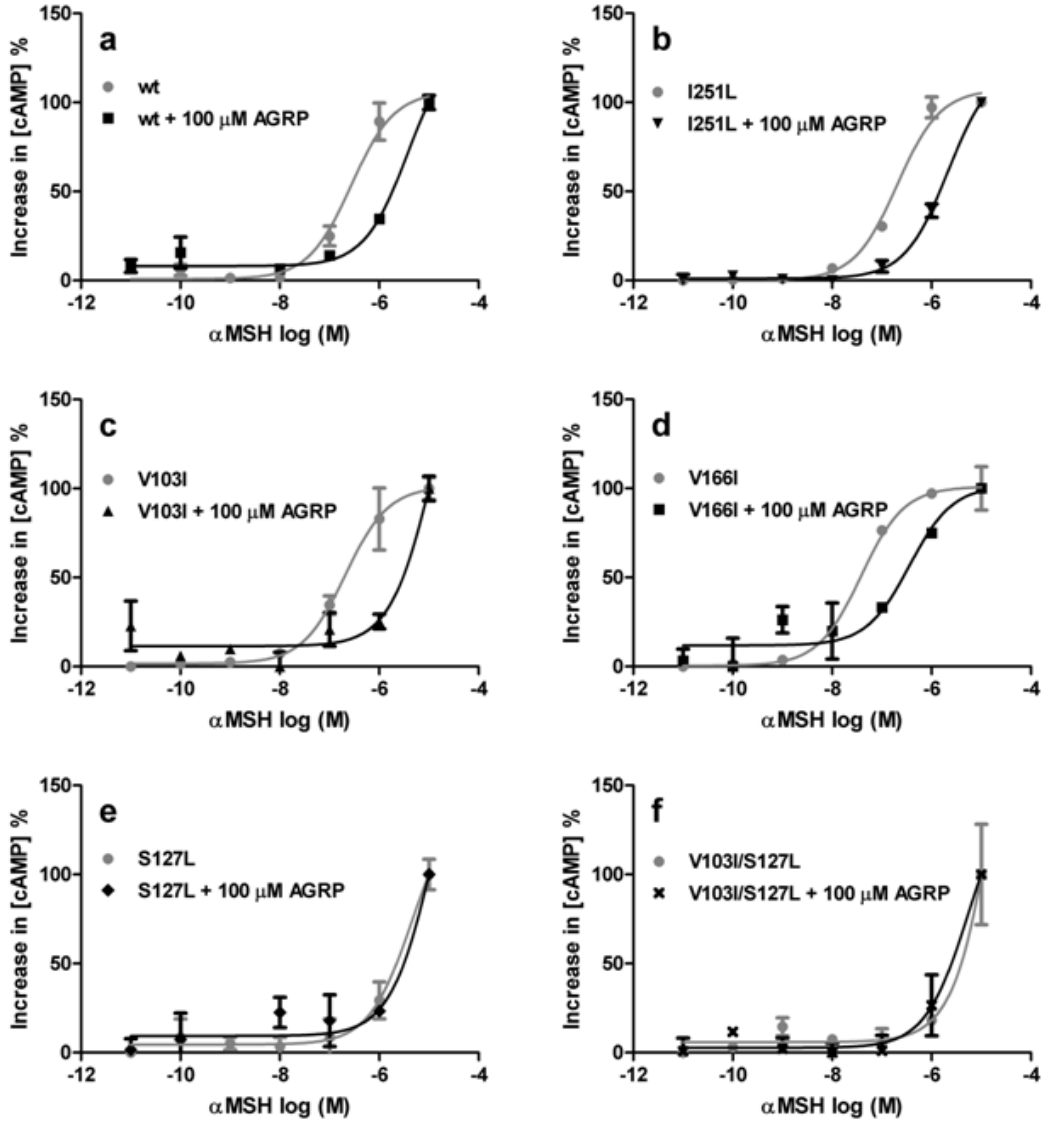

Figure 5. Cyclic adenosine monophosphate (cAMP) response curves of melanocortin 4 receptor (MC4R) mutants in BHK (baby hamster kidney) cells with addition of 100 μM agouti-related protein (AGRP). cAMP concentration is expressed as percentage with the basal level set to 0% and the highest level in each data set to 100%. a – wt, b – I251L, c – V103I, d – V166I, e – S127L, f – V103I/S127L MC4R variants

Discussion

In this study we explored the role of genetic variations in *MC4R* as risk factors for development of morbid obesity. We genotyped extremely obese (BMI > 39.28 kg/m^2) and normal weight controls (n = 760) for four common BMI associated polymorphisms and found correlation for two *FTO* SNPs - rs11642015 and rs62048402 with obesity, however, no association was detected for rs17782313 located in *MC4R* locus. The sequencing of the complete *MC4R* coding region followed by functional recombinant expression studies of four discovered nonsynonymous receptor variants and one double mutant revealed that the V166I substitution might cause hyperactive satiety signal transduction despite occurrence in morbidly obese individual, while S127L could be the reason for extreme adiposity due to functional impairment of the MC4R.

We chose to genotype the rs17782313 because it has demonstrated strong association with BMI in meta-analysis of several GWAS (Consortium, 2007; Loos *et al.*, 2008). We found no association of rs17782313 with extreme obesity, but the same was not true for SNPs in gene *FTO*, the most commonly associated obesity gene. Thus minor alleles of two SNPs recently identified by massive sequencing (Sallman Almen *et al.*, 2012) were also more frequent among cases in our study, however, with smaller effect. Interestingly, other association studies of rs17782313 have correlated this SNP with quantitative traits like: body mass index (BMI), waist circumference and body weight etc. (Hardy *et al.*, 2010; Haupt *et al.*, 2009), but as we did not have normal distribution in our study groups and due to specific selection of the extremely adipose sample pool, we were not able to calculate association with quantitative parameters. It could be suggested that stronger correlation between rs17782313 and quantitative phenotypes as opposed to qualitative parameters (obesity) might exist. Nonetheless, literature data indicate that the *MC4R* risk allele has less impact on BMI than *FTO* polymorphism (0.22 kg/m^2 and 0.36 kg/m^2 increase in BMI, respectively) (Frayling *et al.*, 2007; Loos *et al.*, 2008), and probably *FTO* SNPs have larger effect on pathogenesis of obesity than common variation of *MC4R*.

We also assessed the possible role of the rare genetic factors as a cause of extreme obesity in our study group. We thus sequenced the coding part of *MC4R* in all subjects to investigate how many of those additional genetic factors may be attributed to nonsynonymous changes in *MC4R*. In order to evaluate the functional

consequences of these substitutions we tested cell surface expression and signalling potency of all these variants. We also discovered a new variant V166I beyond finding several previously known mutations. Interestingly, the V166I substitution, despite the significant decrease in receptor quantity on the cell surface, caused increased cAMP response after αMSH stimulation and consequently a significantly higher EC_{50} value compared to the wt MC4R (Table 3, Figure 4). At the same time V166I lead to decreased inhibition by the antagonist AGRP compared to the wt MC4R (Table 3, Figure 5). In physiological terms this would mean that V166I hyperactivity leads to increased satiety signal caused by αMSH activation, while inefficient AGRP inhibition fails to trigger food intake. It is intriguing since we found this alteration in the group of severely obese subjects, therefore, it is difficult to predict what is the balance between the low cell surface expression and the increased activity as well as overall impact of this substitution on the food intake *in-vivo*.

The position 166 of the MC4R is located in the inner part of 4th transmembrane domain of the receptor (Figure 1) and, therefore, may interact with G proteins. Other nonsynonymous substitution described in this region is R165W (Lubrano-Berthelier *et al.*, 2006). This alteration is demonstrated to significantly reduce quantity in plasma membrane of the MC4R, which is similar to our data, however, the activity of the receptor is reduced by approximately 8-fold (Lubrano-Berthelier *et al.*, 2006). Thus the increased cAMP signalling of V166I receptor variant despite the low cell surface expression might suggest that the isoleucine residue at the position 166 promotes increased G protein coupling with the receptor, contrary to the described effects of R165W variant.

Two other nonsynonymous variations V103I and I251L found in our study cohort have been previously reported as protective to obesity risk (Mirshahi *et al.*; Stutzmann *et al.*, 2007; Wang *et al.*, 2010; Young *et al.*, 2007). We also found that the V103I variant was more frequent in the control group (n=12) compared to cases (n=8), but this result was not statistically significant. In our study we detected no significant differences in EC_{50} values for the V103I and I251L mutants when stimulated with αMSH, but addition of AGRP induced stronger inhibition of cAMP accumulation for the V103I variant compared to the wt MC4R. This result is different to findings of Xiang and colleagues, where decreased binding of antagonist AGRP

was observed for the V103I (Xiang *et al.*, 2006), however, it is important to note the methodological difference as we used cAMP measurement instead of binding.

The S127L variant of the *MC4R* has been previously described to have significantly decreased membrane trafficking and functional activity (Calton *et al.*, 2009; Fan *et al.*, 2009) which is in agreement with our results. Interestingly, we discovered two individual carriers with both the V103I and S127L substitutions and we explored the functionality of this double mutant. Our results demonstrate that together V103I/S127L have higher increase in relative cAMP response than S127L alone (Figure 4. d) and considering the curve shifts of the normalised data (Figure 4. b), it is obvious that S127L has lower potency compared to the double substitution receptor. This suggests that the V103I mutation modulates the effect of S127L on MC4R activity. The V103I could increase both potency and efficacy of the receptor which is in turn down-regulated by S127L. We discovered three carriers of the S127L substitution in the group of extremely obese patients, but none in the controls. Therefore, taking in consideration the important alterations in signal transduction caused by this variant, we propose that the genetic variant S127L is the most frequent MC4R mutation leading to morbid obesity in the Latvian population.

At the beginning of study design, we proposed two plausible scenarios: firstly, that those with the more pronounced obesity phenotype show stronger association with the common SNPs and secondly, that rare genetic factors are more likely to be responsible for development of morbid obesity and that these rare factors then may mask and weaken the impact of the common SNPs. We did not find stronger association with the common obesity variants, but we detected one alteration, S127L, that probably can promote increased food intake in three adipose subjects. However, this would unlikely shift the association strength within the study group. This suggests that indeed rare genetic variants can conceal effects of the common SNPs. But these common variants may be located in different obesity related loci, in this case not only *MC4R*. The combination of such rare and common variants could be important for the extreme obesity cases.

The strength of this study is that we studied both common variation and rare mutations as causative factors for morbid obesity, furthermore, functional expression experiments allowed us to demonstrate the possible role of nonsynonymous *MC4R* variations on receptor physiology. The limitations of this study are: 1) we do not have

sufficient power to detect smaller effects of rs17782313 and 2) we have high frequency of cardiovascular conditions and diabetes among the controls, that both can be explained by specific study group selection from LGDB that contains restricted number of morbidly adipose individuals from which we chose the extremely obese subjects. Large proportion of the LGDB participants have been recruited through hospitals, therefore, the frequency of these diseases in normal weight subjects might be higher than in the general population.

In summary we have assessed the role of the *MC4R* genetics as possible cause of extreme obesity in the Latvian population. We detected association with common genetic variants of *FTO* (rs11642015 and rs62048402), but not with *MC4R* SNP (rs17782313). The functional studies of novel MC4R substitution V166I revealed that it causes hyperactivity of the receptor. We also propose that nonsynonymous alteration S127L can lead to adiposity development via abolishment of MC4R functions.

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Disclosure

All authors declare no conflict of interest.

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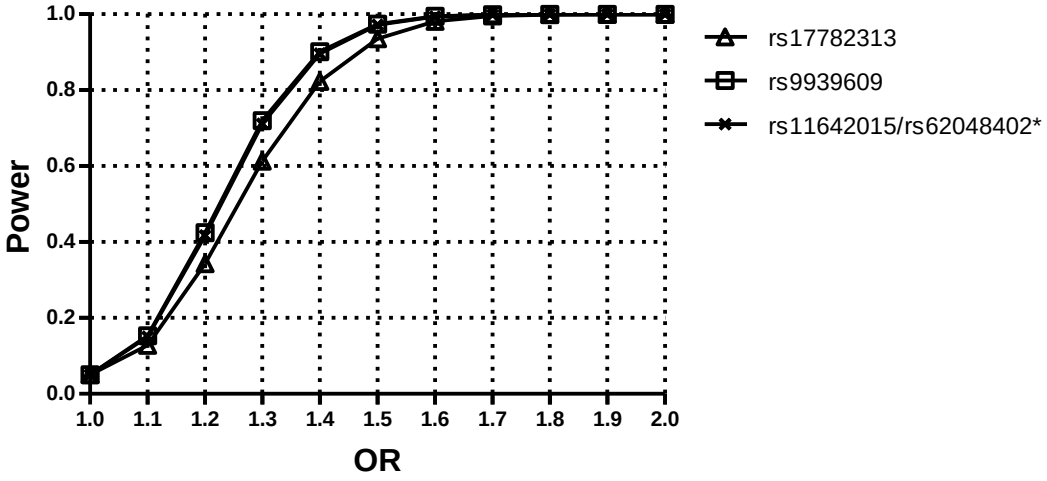
Supplementary Material 1 **Primers used in this study**

Primer name	Primers for amplification of the coding region of MC4R
MC4R pcr Fw1	GGAGGAAATAACTGAGACG
MC4R pcr Rs1	CCAATCAGGATGGTCAAG
MC4R pcr Fw2	TATGCTGGTGAGCGTTTC
MC4R pcr Rs2	TTCAGGTAGGGTAAGAGT
Primer name	**Primers for sequencing of the MC4R**
MC4R seq Fw1	CTGAGACGACTCCCTGAC
MC4R seq Rs1	CCAATCAGGATGGTCAAG
MC4R seq Fw2	GGATCAGAAACCATTGTC
MC4R seq Rs2	TGTTCCTATATTGCGTGC
Primer name	**Primers for site-direct mutagenesis of the MC4R**
MC4R V103I Fw	CTGGTGAGCGTTTCAAATGGATCAGAAACCATTATCATCACCCTATTAAACAGTACAGATACGG
MC4R V103I Rs	CCGTATCTGTACTGTTTAATAGGGTGATGATAATGGTTTCTGATCCATTTGAAACGCTCACCAG
MC4R S127L Fw	CAGTGAATATTGATAATGTCATTGACTTGGTGATCTGTAGCTCCTTGCTTGCATCC
MC4R S127L Rs	GGATGCAAGCAAGGAGCTACAGATCACCAAGTCAATGACATTATCAATATTCACTG
MC4R V166I Fw	CCAGTACCATAACATTATGACAGTTAAGCGGATTGGGATCATCATAAGTTGTATCTGGGCAGC
MC4R V166I Rs	GCTGCCCAGATACAACTTATGATGATCCCAATCCGCTTAACTGTCATAATGTTATGGTACTGG
MC4R I251L Fw	CCAATATGAAGGGAGCGATTACCTTGACCATCCTGCTTGGCGTCTTTGTTGTCTGCTGGGCC
MC4R I251L Rs	GGCCCAGCAGACAACAAAGACGCCAAGCAGGATGGTCAAGGTAATCGCTCCCTTCATATTGG

Supplementary Material 2 **Characteristics of the genotyped SNPs**

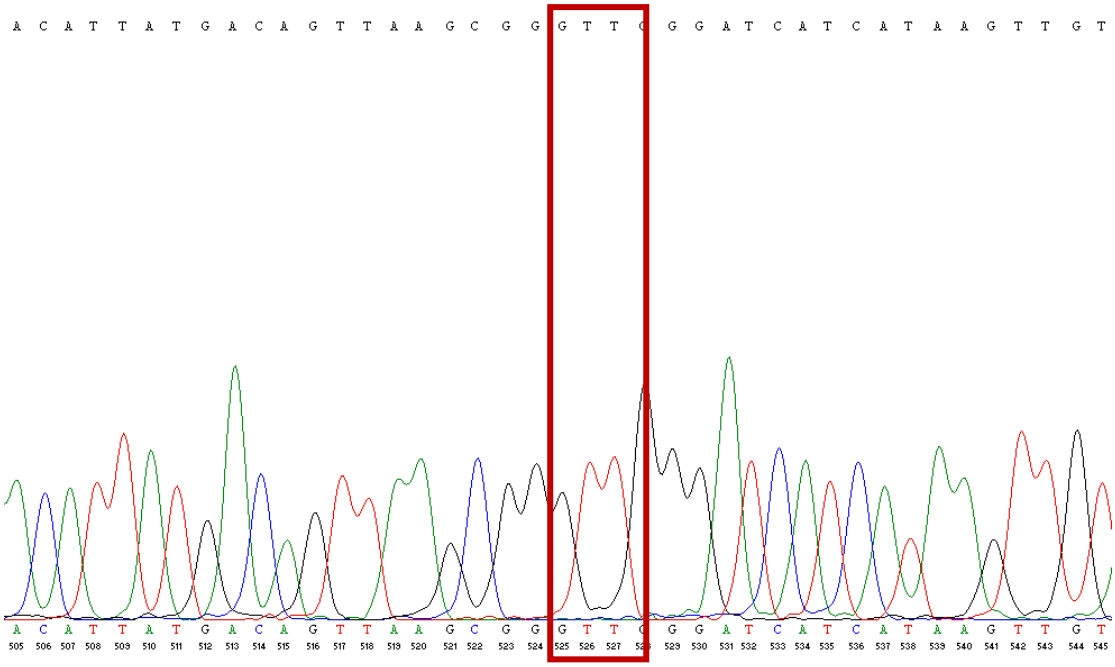
rs code	Genome position	Minor allele	MAF	MAF controls	MAF cases	Genotyping success (%)	H-W test *p*-value
rs17782313	54560394	C	0.196	0.189	0.203	99.5	0.248
rs9939609	39707357	A	0.487	0.463	0.511	100	0.942
rs11642015	39689324	T	0.493	0.463	0.524	99.5	0.772
rs62048402	39690053	A	0.493	0.463	0.524	99.3	0.772



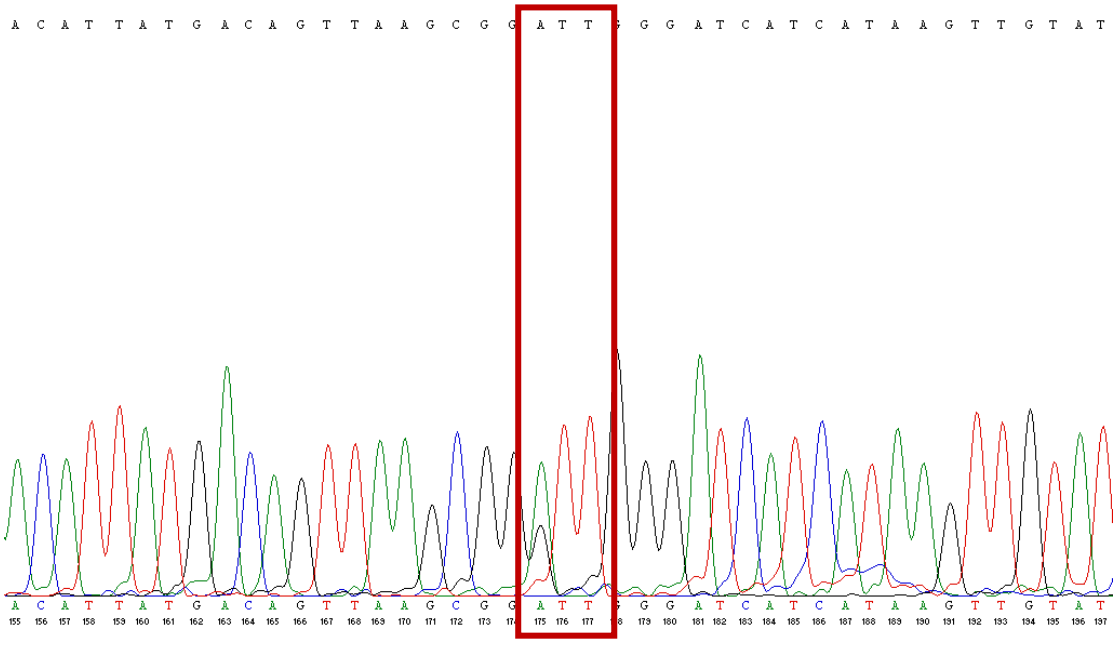
Supplementary Material 3 **Power to detect association OR for studied SNPs.**

* rs11642015 and rs62048402 are presented together due to complete linkage disequilibrium between them

a

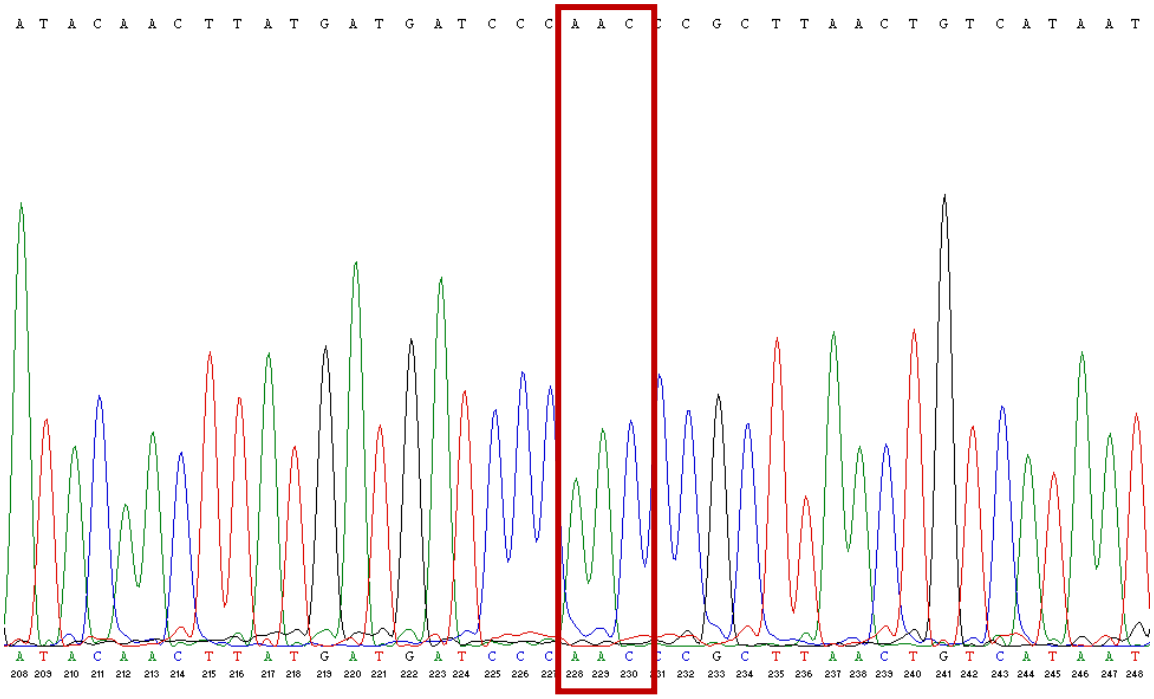


b

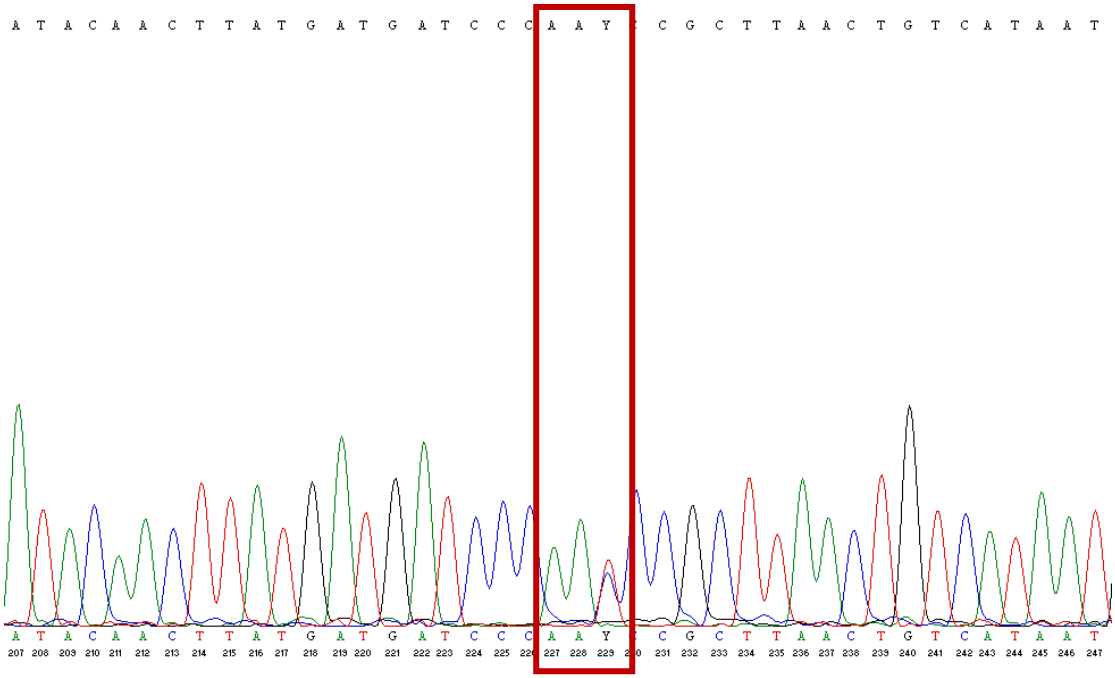


Supplementary Material 4 **Forward sequence of nucleotides coding for 166 amino acid position of the MC4R.** (a) wild-type MC4R, (b) V166I MC4R.

c



d



Supplementary Material 4 **Reverse sequence of nucleotides coding for 166 amino acid position of the MC4R.** (c) wild-type MC4R, (d) V166I MC4R.

4 DISCUSSION

The results of the studies in this thesis present the comprehensive research of purinergic and melanocortin receptors representing two distinct subgroups of GPCRs. We have demonstrated that these two distinctly different receptor types can be expressed in a yeast model system and functionally characterised. Furthermore, we studied the possible involvement of *MC4R* and *P2RY1* in development and progression of different multifactorial diseases.

Previous functional studies have considered MC4R to be a crucial factor of body mass regulation (Huszar *et al.*, 1997). It is also recognised as one of the most important monogenic factors of morbid obesity (Mergen *et al.*, 2001; Vaisse *et al.*, 2000). The *MC4R* locus and its common variants, however, seem to be less important determinants of obesity than the fat mass and obesity-associated protein gene *FTO*. Although mutations in *MC4R* can explain a limited number of obesity cases, the impact of common SNPs on the pathogenesis of extreme obesity is unclear. We therefore attempted to investigate morbid obesity (BMI > 39 kg/m^2) and related phenotype association with both the common polymorphism near *MC4R* and changes in the coding sequence of *MC4R*.

The Genome Database of the Latvian Population (LGDB) constitutes almost 1% of the entire population of Latvia. Consequently, the selected group of the 380 most obese patients from the LGDB represents a significant proportion of all obesity cases in Latvia. The results of this study are discussed in detail in Chapter 3.4. The SNP rs17782313 was selected for analysis based on a meta-analysis of several GWAS (Consortium, 2007; Loos *et al.*, 2008) in which it displayed the strongest association with BMI. However, our data showed no association with obesity in morbidly adipose individuals. This result could indicate that stronger genetic factors than common polymorphisms underly the pathogenesis of extreme obesity. To test whether the SNPs at the other obesity gene *FTO* locus, displayed the same lack of association we genotyped three SNPs from *FTO* in our study cohort. Contrary to the analysed polymorphism in *MC4R*, we observed association of obesity with *FTO* polymorphisms rs11642015 and rs62048402, the recently identified strongly associated obesity variation within a transcription regulatory site (Sallman Almen *et al.*, 2012). The commonly studied *FTO* SNP rs9939609 was close to the border of

significance (P = 0.066; odds ratio (OR) = 1.21 [0.99-1.48]). This result indicated that although with the reduced effects, the impact of common SNPs (at least at the *FTO* locus) was significant, possibly accompanied with the influence of other genetic or nongenetic factors.

For other genotype associations, GWAS findings are not consistently replicable in other populations (Ioannidis, 2007). Since GWAS usually reveals genetic association with OR not greater than 1.5, this demonstrates that effects of the determined genotype are not essential for the disease development, but more likely serve as one of a range of contributing factors leading to medical dysfunctions. Thus, the lack of association of *MC4R* rs17782313 in our study might be explained by the insufficient power due to small sample group size. GWAS replications have been done in populations of various origins with different health status and lifestyles that contribute with genetic polymorphisms to multifactorial diseases like adiposity (Jacobsson *et al.*, 2008; Ng *et al.*, 2010). Previous reports have linked rs17782313 mainly with quantitative parameters such as body mass index (BMI), waist circumference, and body weight (Hardy *et al.*, 2010; Haupt *et al.*, 2009), while we compared the presence of genotype between the lean and obese groups. The specificity of our study group including the extreme end approach did not allow us to perform the parametric analysis using BMI as quantitative value for association. Other possible explanation for lack of association is that stronger rare genetic factors are responsible for development of morbid obesity and these factors then masks and weakens the impact of common SNPs.

Functional mutations within *MC4R* could be among such stronger factors that might influence obesity. We therefore sequenced all *MC4R* coding region in our study group. We identified four heterozygous nonsynonymous substitutions in *MC4R*: V103I, S127L, V166I and I251L in morbidly obese subjects. Interestingly, two people were double mutants for both V103I and S127L mutations. To investigate the functionality of these MC4R variants, mutant receptor constructs were made and expressed in a mammalian cell system (detailed discussion in Chapter 3.4). Two previously reported substitutions, V103I and I251L, demonstrated no significant effect on MC4R functionality, although V103I had a slightly decreased relative cAMP response compared to the wt receptor when activated with αMSH. Interestingly, these alterations have been found to be protective against obesity in several genetic

association studies (Mirshahi *et al.*, 2011; Stutzmann *et al.*, 2007; Wang *et al.*, 2010; Young *et al.*, 2007). In our study cohort we also detected V103I more often in controls (n=12) than in obese subjects (n=8), but the difference was not statistically significant. Our results and other reports (Hinney *et al.*, 2003; Ho *et al.*, 1999; Xiang *et al.*, 2006) found no essential functional inclinations in MC4R signalling when stimulated by agonist. However, it has been reported that V103I significantly lowers affinity for the natural antagonist AGRP (Xiang *et al.*, 2006), that suggests that natural orexigenic signal reception (antagonist AGRP) by MC4R for V103I carriers is reduced, while affinity for the satiety signal (agonist αMSH) remains. Therefore, transduction via receptor is shifted toward satiety and might lead to decreased risk for obesity. Our results, however, demonstrated that addition of AGRP induced stronger inhibition of cAMP concentration for the V103I variant compared to the wt MC4R. It should be noted that these results are no comparable with literature as we used cAMP accumulation measurement instead of binding assay.

Originally, the substitution V166I exhibited a significantly higher cAMP response then wt MC4R, despite the poor cell surface trafficking. One may suggest that V166I alteration affects G protein coupling to the receptor, since position 166 of MC4R is on the edge of the inner cell surface and could interact with G protein. For example, V166I could increase binding of the G protein to the receptor and therefore cause MC4R hyperactivity after αMSH stimulation. In physiological terms, hyperactivity of the MC4R means an increase in satiety signal that would be protective against obesity. These findings were complemented by antagonist experiments in which AGRP inhibition was lower than for wild type also indicating the shift toward decreased food intake. As we detected V166I substitution in extremely adipose person, it is difficult to evaluate how low cell surface expression interact with the increased receptor activity and what physiological consequences it causes in living organism.

The substitution S127L has been studied previously and it is reported to decrease membrane trafficking and signal transduction (Calton *et al.*, 2009; Fan *et al.*, 2009). In our study cohort, we discovered two individuals with both S127L and V103I substitutions. We described the effect that V103I had on S127L-induced changes in MC4R functionality. Our data demonstrated that V103I was able to increase potency and efficacy of the MC4R that is down-regulated by S127L. Since

we have found S127L only in severely obese individuals and, furthermore, functional experiments suggest that physiological role of this alteration is increased food intake, we propose that S127L is the most frequent *MC4R* mutation that cause extreme adiposity in the Latvian population. However, we only detected S127L substitution in three obese subjects and this mutation alone cannot decrease the association strength for rs17782313 within the study group, as was proposed. Overall, our results demonstrate that rare variants are the more probable determinants of morbid obesity than common polymorphisms, but these individual mutations are not located only in *MC4R* locus, but in other obesity related genes as well. For deeper comprehension of genetics underlying the development of extreme adiposity targeted gene sequencing of obesity related loci or complete genome sequencing of subjects with pronounced inherited adiposity phenotypes is required.

For the genetic association study of complete *P2RY1* locus (results discussed in Chapter 3.2) we used more extensive approach in common SNPs analysis by simultaneous correlation of the polymorphisms not only with MI but also related phenotypes to adjust for possible interaction of all involved traits. We explored association of polymorphisms covering the entire gene *P2RY1* locus with seven parameters emphasizing cardiovascular events. In this genetic study, a candidate gene approach was used and a target gene was selected based on the physiological role of P2Y1R in blood coagulation processes. These were well demonstrated in previous studies (Leon *et al.*, 1999), but genetic variations were not shown to influence any phenotype. Candidate gene association is based on the hypothesis of a presumed link between genotype and phenotype based on known function, while replication of the association depends more on data from GWAS or other genetic studies. Both study designs have advantages and drawbacks. GWAS and replication of GWAS can study genome regions not previously annotated functionally. After discovery of significant association, explanation of mechanisms underlying the genotype influence on phenotype can be difficult. Furthermore, a potential SNP could be in strong linkage disequilibrium with other polymorphisms that expand the association region enormously, making understanding of disease development more difficult. However, the advantage of GWAS-related methods is that the results can indicate novel functions of proteins that in turn can help in understanding the influence of the genotype on phenotypic traits.

Other candidate genes of blood clotting factors have been studied in relation to heart disease development (Ken-Dror *et al.*, 2010; Mo *et al.*, 2011). Our study, however, did not find any association of tagSNPs in the *P2RY1* locus with MI. Other rare polymorph variants of P2Y1R might still be associated with heart condition development, but our ability to detect such variants was limited by the use of tagSNP genotyping. Intriguingly, our results demonstrated a tendency toward late-onset MI in the *P2RY1* polymorphism carriers (Chapter 3.2). This fits well with the hypothesis that the observed effect could be caused by lower blood coagulation intensity in these individuals and decrease thrombus formation potential, delaying heart condition progression. However, more studies are needed to investigate this hypothesis.

We explored *Saccharomyces cerevisiae* expression systems by studying two different types of receptors purinergic and melanocortin. GPCR expression in yeast has been compared to mammalian cell models (Beukers *et al.*, 2004; Pausch *et al.*, 2004). Each has advantages and drawbacks, but for specific experimental needs one system might be advisable over the other. We suggested that expression of P2Y12R in yeast could be more sensitive and specific than in mammalian cells (Chapter 3.3) for several reasons. First, different kind of purines and their signalling systems are abundant in mammalian cells. This might cause background activity of the receptor. Second, as mammalian cells express other GPCRs besides the desired receptor, oligomerization could occur to shift the activation signal in a specific manner. In yeast, the model system is isolated from the impact of other receptors. The oligomerization of MC4R has been indirectly implicated in the cause of monogenic form of severe obesity in heterozygous case (Biebermann *et al.*, 2003). In addition to oligomerization, other physiological mechanisms can adjust the final signal transduction via GPCRs, for example, ligand effects on receptor-G protein binding, proteins that regulate G protein activity, and signalling of GPCRs via non-G protein pathways (Cao *et al.*, 2004; Ladds *et al.*, 2003; Zhai *et al.*, 2005). This indicated that all features influencing GPCR signalling and trafficking must be considered not only in functional but also genetic receptor research. Furthermore, yeast and mammalian model systems can complement each other in these studies.

One of the most relevant factors to consider in using *Saccharomyces cerevisiae* for GPCR research is ligand size. Yeast cells have a dense polysaccharide-rich cell wall that can affect migration of the ligand to the receptor expressed on the

plasma membrane. However, we demonstrated that even ACTH with 39 amino acids (Fw = 4541) can successfully cross the yeast cell wall and activate the receptor (Chapter 3.1). Another disputable point concerning the receptor ligand is stability. For P2Y12R activation, ADP was used, which is believed to be unstable in biological systems. To assess this, we performed a detailed analysis of P2Y12R activation by ADP and the chemically stable synthetic compound 2MeSADP. Our results indicated functional activity of the receptor similar to previous reports (Pausch *et al.*, 2004; Zhang *et al.*, 2001), showing that hydrolysis of ADP did not affect the detected activity of P2Y12R.

For functional studies in this thesis for both MC4R and P2Y12R, we used site-directed mutagenesis to characterise distinct amino acids in the receptor protein to determine their role in receptor activity. The results are discussed in Chapters 3.1 and 3.3. Usually, site-direct mutagenesis of GPCRs involves substitution of specific amino acids with other amino acids that are chemically neutral, although sometimes substitution with amino acids of opposite properties is used (Hoffmann *et al.*, 2008; Yang *et al.*, 2000). This approach does not completely describe the functionality of the studied position. We generated and characterized randomised libraries of selected residues and found that any substitution of D126 in the MC4R and K280 of the P2Y12R completely abolished receptor function. A three-dimensional model of P2Y12R suggested that K280 is a fixating switch that fastens ADP into the ligand binding cavity between the TM helices of the receptor. Any substitution of the Lys in this position even with chemically related Arg, disabled the receptor.

Similar results were observed for D126 of the MC4R where even substitution with another acidic residue (Glu) abolished receptor signalling. Interestingly, we observed that some substitutions such as Leu and Glu had higher background activity than others such as Phe, suggesting that this MC4R position might be involved in constitutive activity of the receptor. To exclude possible effects of the yeast physiological state we performed a second round of random receptor plasmid isolation and retransformation and obtained the same results. The constitutive activity of MC4R has been previously described (Srinivasan *et al.*, 2004). Beukers and colleagues demonstrated that random point mutations of other GPCRs can cause receptor constitutive activity (Beukers *et al.*, 2004). Intriguingly, most of these substitutions were neutral, aliphatic or acidic amino acids, leading to the overall

observation that constitutive activity of the GPCRs can be caused by definite biochemical interactions of amino acid residues at specific sites. Further experimental studies are needed to analyse this hypothesis.

Besides, the K280 residue analysis, P2Y12R revealed interesting facts about other receptor positions as well. Substitutions of E181 pointed out that hydrophility in this position is preferred for better receptor activation. Since the E181 is located in EL of the P2Y12R it is unlikely that this residue is involved in formation of ligand binding pocket even though the ELs of GPCRs have been demonstrated to guide and fix receptor ligand into it's binding site. Detailed description of the ligand binding to the P2Y1R has been done specifying the meta-binding sites that direct ligand into main binding cavity (Moro *et al.*, 1999). It is possible that this kind of interaction occurs also in other P2YRs, furthermore, acidic amino acids like Glu or Asp are common in EL2 of most P2YRs indicating possible involvement in signalling mechanisms of this receptor group. Two other P2Y12R residues analysed were R256 and R265. There is definite evidence in the literature that these positions affect signalling properties of P2Y12R (Cattaneo *et al.*, 2003; Hoffmann *et al.*, 2008; Mao *et al.*, 2010). We observed that substitutions of R256 can dramatically decrease or completely abolish functionality of the P2Y12R. Since R256 residue is located in the upper part of TM helix of the receptor the most probable way in which it can affect P2Y12R signalling is its involvement in the formation of ligand binding pocket. On the contrary R265 mutant variants that are also located in the upper part of TM domain, did not show such dramatic decrease in receptor activity as R256 receptor variants. However, tendency can be observed that statistically significant shifts in EC_{50} values were detected for Ala, Leu and Ile variants of R265 mutant library, indicating that aliphatic and hydrophobic properties are not preferable at this site for functional integrity of the receptor upon ADP activation (Chapter 3.3, Table 1).

Inhibition experiments with antagonist AR-C66096 showed no strict patterns of biochemical properties required at the studied positions for its functional inhibition of P2Y12R. But as all ligand activity curves indicate, some randomised receptor variants had greater inhibition shift than others (Chapter 3.3, Figure S2) suggesting that these positions are involved in AR-C66096-P2Y12R binding. However, to gain full understanding of the interaction process, further experiments are needed.

We have analysed functional results of the P2Y12R mutant libraries activation using two bioinformatic methods: QSAR and three-dimansional modelling. These methods supplement each other and enable comprehensive characterization of practical data. QSAR is used more often in targeted ligand design in order to predict binding of series of novel compounds and save time and expenses over laboratory experiments (Hao *et al.*, 2011). Some studies, however, use QSAR to gain deeper knowledge of the protein regions involved in different biological interactions (DeVore *et al.*, 2009; Wang *et al.*, 2009a). We used QSAR to characterise type of physiochemical properties of amino acids required for interaction of P2Y12R and ADP. The advantage of using QSAR is that, we were able to successfully correlate our E181 and R256 mutant library experimental data with particular physiochemical properties including charge, steric/bulk properties, polarity, electronic effects, rigidity and flexibility, and to obtain mathematical models that characterise and quantify the ligand binding site that could not be done by manual analysis of experimental results alone. These data are essential for novel ligand design as it helps predict ligands that are prone to interact with the receptor and should be included in functional studies to encompass full complexity of physiochemical properties in biological systems. In addition to QSAR, another technique that could be used in similar research is proteochemometrics that could integrate both target receptor and ligand determinants (Lapins *et al.*, 2008).

For generation of a three-dimensional P2Y12R structure model we used homology modelling to observe possible interactions of ADP with the studied residues of the receptor to determine those involved in ligand binding. The hydrophobic nature of GPCRs makes it difficult to obtain soluble protein isolates, hindering crystallization and X-ray structure development. Therefore, homology modelling on the templates of the resolved GPCR structures is often used. The template selection for homology modelling greatly affects the final model including how well the obtained model corresponds to the structure of the *in-vivo* receptor. Distinct differences between available templates have been characterised (Tate *et al.*, 2009) and using the best fitting template is important. We used the adenosine 2A receptor (A2aR) 2.6 Å resolution model as a template (Jaakola *et al.*, 2008), because at the time, this was the most closely related crystal structure to P2Y12R. In addition to the chemical properties of the natural agonist of the A2aR, adenosine is similar to

ADP, so the ligand binding pockets of these receptors could share considerable structural resemblance.

In summary, this thesis conducted complex genetic and functional studies of melanocortin and purinergic receptor signalling and outlined important aspects of a yeast expression system and candidate gene association approach in multifactorial disease research.

5 CONCLUSIONS

1. Substitutions of the D126 position of MC4R with any amino acid abolish ligand-mediated signal transduction via the receptor.
2. Neutral, aliphatic or acidic substitutions at the 126 position of MC4R lead to increased background constitutive activity, while aromatic hydrophobic residue substitution variants have decreased constitutive activity.
3. The gene *P2RY1* locus is not associated with myocardial infarction, body mass index, type 2 diabetes, *angina pectoris*, hypertension, hyperlipidemia, atrial fibrillation or heart failure in the population of Latvia.
4. The *Saccharomyces cerevisiae* model system has been successfully used to express and functionally characterise both GPCRs binding small molecules (P2Y12R) and large peptides (MC4R).
5. Hydrophobic residues at position E181 of P2Y12R decrease functional activity of the receptor. Any substitution of the R256 residue dramatically decreases P2Y12R signalling and the functional activation of P2Y12R. R265 random variants proved the importance of this position in the structural integrity of the receptor.
6. Residue K280 serves as a fixing switch in the P2Y12R ADP binding pocket.
7. *MC4R* polymorphism rs17782313 is not associated with severe adiposity when compared with normal weight controls, while *FTO* polymorphisms rs11642015 and rs62048402, but not rs9939609, were significantly associated with obesity in the same cohort.
8. V103I and I251L variants of MC4R had functional properties similar to the wild type receptor when stimulated with αMSH, but V103I demonstrated stronger AGRP inhibition.
9. S127L had a significantly decreased receptor quantity at the cell surface and decreased functional activity. In the double mutant V103I/S127L, the substitution V103I appears to modulate the effects of S127L.
10. V166I alteration in MC4R causes decreased receptor quantity in the plasma membrane, however, this receptor variant has a significantly higher cAMP response upon stimulation with αMSH, but AGRP inhibition is decreased compared to wild type MC4R.

6 MAIN THESIS FOR DEFENCE

1. Position D126 of MC4R is crucial for functional activity of the receptor and is involved in constitutive activation of the receptor.
2. Polymorphisms in *P2RY1* do not affect development of cardiovascular conditions, despite its role in blood coagulation mechanisms.
3. Hydrophilic properties of the residues in extracellular loop 2 of the P2Y12R are relevant for functional activity of the receptor.
4. K280 of the P2Y12R is an essential determinant of the P2Y12R ADP binding pocket.
5. *Saccharomyces cerevisiae* can be used as an expression system for functional research on GPCRs.
6. Common *FTO* polymorphisms are more significant determinants of morbid obesity than SNP at the *MC4R* locus.
7. The originally discovered nonsynonymous substitution V166I in the coding region of the gene *MC4R* might cause a hyperactive satiety signal reception.
8. The nonsynonymous substitution S127L is the most frequent functional variant of *MC4R* causing morbid obesity.

7 LIST OF ORIGINAL PUBLICATIONS

1. Ignatovica V, Petrovska P, Fridmanis D, Klovins J. Expression of human melanocortin 4 receptor in *Saccharomyces cerevisiae*. *Cent Eur J Biol*. 2011; 6(2):167-175.
2. Ignatovica V, Latkovskis G, Peculis R, Megnis K, Schioth HB, Vaivade I, Fridmanis D, Pirags V, Ergis A, Klovins J. Singe nucleotide polymorphisms of the purinergic 1 receptor are not associated with myocardial infarction in a Latvian population. *Mol Biol Rep*. 2012; 39(2):1917-25.
3. Ignatovica V, Megnis K, Lapins M, Schioth HB, Klovins J. Identification and analysis of functionally important amino acid residues in the human purinergic 12 receptor using yeast *S. cerevisiae* expression system. *FEBS J*. 2012; 279(1):180-91.
4. Ignatovica V, Petrovska R, Kalnina I, Vaivade I, Fridmanis D, Tarasova L, Peculis R, Schioth HB, Klovins J. Analysis of common and rare MC4R variants and their role in extreme form of obesity. *Submitted to Obesity.*

8 APPROBATION OF THE RESEARCH

8.1 PUBLISHED THESIS (International)

1. Ignatovica V, Klovins J. Functional selection of randomized library of MC4 receptor in yeast *Saccharomyces cerevisiae.* The FEBS Journal, Volume 275, Supplement 1, June, 2008, p451.
2. Ignatovica V, Peculis R, Kalnina I, Kolvins J. Expression of human adenosine A2a and human purinergic P2Y1 and P2Y12 receptors in yeast *Saccharomyces cerevisiae* for screening of novel therapeutics. 12th International Congress on Yeasts, Kyiv, Ukraine, 2008, August 11th – 15th.
3. Ignatovica V, Peculis R, Kalnina I, Kolvins J. Expression of adenosine and purinergic receptors in yeast *Saccharomyces cerevisiae* for screening of novel therapeutics. European Journal of Medical Research, Volume 13, Supplement 1, September 29, 2008.
4. Ignatovica V, Kalnina I, Peculis R, Klovins J. Functional selection of randomized libraries of G-protein coupled receptors in yeast *Saccharomyces cerevisiae* for development of novel therapeutics. 27th International Specialized Symposium on Yeasts, Paris, France, 2009, August 26th – 29th. Abstract Book, Institut Pasteur Paris, 2009, p227.
5. Ignatovica V, Megnis K, Klovins J. Functional analysis of P2Y12 randomized libraries in yeast *Saccharomyces cerevisiae*. The FEBS Journal, Volume 277, Supplement 1, June, 2010, p122.
6. Ignatovica V, Megnis K, Klovins J. Functional characterization of P2Y12 receptor for future development of novel antiplatlet therapeutics. Pathophysiology of Haemostasis and Thrombosis, Vol 37 (supp 1), 2009-2010, p-A146.
7. Ignatovica V, Megnis K, Lapins M, Schioth HB, Klovins J. Characterization of functionally important amino acid residues in the human purinergic 12 receptor using yeast *Saccharomyces cerevisiae* expression system. 4th Joint German-Italian Purine Club Meeting, Bonn, Germany, 2011, July 22nd-25th. Abstract Book, Universitat Bonn, 2011, p76, PS2.10.
8. Ignatovica V, Megnis K, Lapins M, Klovins J. Functional characterization of the human purinergic 12 receptor using a *S. cerevisiae* model system. International Conference in Pharmacology, Riga, Latvia, 2012, April 20th-21st. Abstract book, 2012, p42, P11.

8.2 PUBLISHED THESIS (National)

1. Ignatovica V, Klovins J. Functional selection of melanocortin 4th receptor random variants in yeast *Saccharomyces cerevisiae.* 66th Scientific Conference of the University of Latvia, Riga, 2008, January-May.
2. Ignatovica V, Peculis R, Kalniņa I, Klovins J. Adenosine and purine receptor expression in yeast *Saccharomyces cerevisiae* for design and construction of novel therapeutics. 67th Scientific Conference of the University of Latvia, Riga, 2009, January-April.
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4. Ignatovica V, Latkovskis G, Peculis R, Megnis K, Pirags V, Erglis A, Klovins J. Characterization of P2Y1R gene locus in association to myocardial infarction in population of Latvia. 69th Scientific Conference of the University of Latvia, Riga, 2011, January-May.
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