Development and Evaluation of Multiplex and High-throughput SNP Analysis for the ABCA1 gene

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Development and Evaluation of Multiplex and High-throughput SNP Analysis for the ABCA1 gene

Doctoral Thesis

by

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“The aim of science is not to open the door to infinite wisdom, but to set a limit to infinite error”.

Berthold Brecht, “The Life of Galileo“

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1 Introduction

The human genome consists of 3.2 billion base pairs. About 99.9% of human DNA sequences are the same across the population, the remaining 0.1% represent our genetic diversity which consists of evolutionarily stable sequence variations in the genome, that usually reflect past mutations. There are different types of genetic variations: base-substitutional polymorphisms, tandem repeat (microsatellite) polymorphisms and a few rare other classes like presence or absence of a region (e.g. rhesus factor gene RHD) or a mobile element (e.g. Alu element). About 90% of all human genetic variations are base-substitutional polymorphisms. These small genetic variations are called SNPs (Single Nucleotide Polymorphisms) and are very common in the human genome. Estimations range from one SNP per 100 to 2,000 nucleotides.[1, 2]

The sequence variations are the basis of differences between chromosomes or chromosomal regions and thus form the different alleles.[3-7] The prevalence of one allele within human beings depends on selection, population history and chance. By definition, the rarer allele should be more abundant than 1% in the general population otherwise the variation is referred to as a point mutation.[1, 3, 4, 8]

In theory, a SNP could have four possible alleles, since there are four types of bases in DNA. However, most SNPs are bi-allelic (>99.9%) and they are not randomly distributed over the whole genome. SNPs with A/G substitutions (and reverse complement T/C) are most prevalent. Since the human genome contains only a few percent coding sequence, the vast majority of SNPs are likely to have little functional consequence. Extrapolations show, that of 10^{6-7} SNPs, 10^{5-6} are gene associated, which means, they are located in or in the near vicinity of genes (especially in introns and the promoter). Around 10^{4-5} are found in the coding regions of genes (exons), of which 10^{3-4} are suspected to result in a relevant phenotype.[7, 9, 10]

SNPs do not cause disease, they can increase the susceptibility or resistance to develop a disease or determine the severity or progression of a disease. One good example for susceptibility to disease related to SNPs is the degenerative disorder Alzheimer's disease. Two SNPs in the apolipoprotein E gene (apo E) have been associated with the age of onset of Alzheimer's disease. These two variations cause amino acid exchanges at codons 112 and 158 and result in three possible alleles: E2 (Cys-112, Cys-158), E3 (Cys-112, Arg-158) and E4 (Arg-112, Arg-158). The apo E4 allele was significantly associated with the development of Alzheimer's disease, whereas the E2 variant has a protective effect.[3, 4, 8, 11-13]

Furthermore, SNPs can alter the body's response to therapeutic drugs. One example for variations that affect the response to therapeutic agents are SNPs in cytochrome P450 enzymes (CYPs). In humans, 49 different CYP isoforms have been identified so far, which play an important role in the oxidative part of drug metabolism. One isoform, CYP2D6, is responsible for the metabolism of about 25% of all drugs, including important agents like antiarrhythmics, ß-blockers and tricyclic antidepressants. More than 70 SNPs in CYP2D6 are known with some of them producing non-functional variants (e.g. CYP2D6*4 and CYP2D6*5), variants with decreased activity (e.g. CYP2D6*10), altered substrate specificity (e.g. CYP2D6*17) and increased activity (CYP2D5*2xN). An individual CYP-SNP-profile could increase the efficiency of medical treatment with therapeutic drugs and reduce or eliminate dose-dependent and dose-independent adverse drug reactions.[6, 14]

These two examples illustrate the motivation for the identification of functionally relevant sequence variations. But how can these SNPs be identified and characterized?

Sequence variations are usually discovered by DNA sequencing and alignment of the sequences obtained of the different individuals and by comparison to database entries. The sequencing of individuals with different ethnical origins and certain phenotypes are more promising to display sequence variations, than randomly selected individuals. Mostly, candidate gene approaches are performed in SNP screening, since whole genome scans are too costly and laborious. Here, candidate genes are selected due to their function, their structure or their chromosomal location and sequencing of these genes or important regions of these genes (e.g. promoter and exons) is performed.

After the discovery of a SNP, frequency determination and association studies in large cohorts are required to investigate the functional relevance of the polymorphism at a statistically reliable level. For a functionally relevant SNP, it has to be shown that one variant of the SNP is significantly more prevalent in one study population compared to another (e.g. patients with a certain disease compared to healthy controls). For this purpose, so-called high-throughput technologies are required to handle the huge amount of analyses. Although the expression "high-throughput" is used very frequently, a useful quantitative definition of this term is scarcely found. The reason for this might be, that technological abilities increase so quickly in this field. Also, different terms like "medium throughput" or "ultra-high-throughput" can be found in the literature, which are mostly used without proper reflection and are usually based on comparison between methods. Therefore, a suitable definition for "high-throughput analysis" would be the fast analysis of a large number of samples in an automated workflow, including the rapid availability of test results.

Usually, not a single, but a number of SNPs are identified to be associated with complex diseases or certain clinical parameters. Therefore, subsequent to the identification of relevant loci, a multiplexing technique, capable of the simultaneous determination of specific SNP profiles would be a great advantage for individual risk assessment or medical treatment.

In the focus of this work is the ATP-binding cassette 1 transporter gene (ABCA1), which has recently been identified as a key player in reverse cholesterol transport. Mutations in ABCA1 are responsible for a rare form of genetic HDL deficiency known as Tangier disease (OMIM 600046), which is characterized by severely diminished plasma HDL-C levels and a predisposition to splenomegaly and frequently cardio-vascular disease (CVD). Since the identification of the ABCA1 gene being responsible for Tangier disease, several causative mutations have been identified in affected individuals, either in homozygous or heterozygous state. These individuals had no detectable HDL and had CVD or hepatosplenomegaly. Interestingly, individuals that are heterozygous for one of the causative mutations have reduced HDL-levels (40-45%) and display some lipid aberrations, thus being at risk for cardiovascular disease [15]. Therefore, it can be assumed that sequence variations in ABCA1 could influence HDL-C levels and development of HDL related diseases, such as arteriosclerosis or premature onset of coronary artery disease. Up to now, 13 sequence variations upstream to the translation initiation site in exon 2 have been reported and more than 60 sequence variations have been found in the coding region of ABCA1.

For the ABCA1 gene as a model, a screening approach for novel SNPs has been performed, a platform for high-throughput analysis has been established and a novel multiplexing technology has been developed.

2 Background

2.1 The ABCA1 gene in HDL metabolism

2.1.1 ABC transporters

The ABCA1 gene belongs to the family of ATP-binding cassette (ABC) transporters. These proteins translocate or regulate the transport of specific molecules across the plasma membrane or intracellular membranes of the endoplasmatic reticulum, the peroxisome, and mitochondria.

ABC transporters consist of two ATP-binding folds, also referred to as nucleotide binding domains (NBD), and two sets of typically six transmembrane domains (TMD). There are also half-size transporters, that contain only one NBD and one set of transmembrane domains, but these transporters usually form homo- or heterodimers to result in a functional transporter. The NBD contain three conserved motifs found in all ATP binding cassette transporters: walker A, walker B and signature (S). The signature, located between the two walker motifs, is characteristic for ABC transporters and is not found in other ATP binding proteins. One group of ABC transporters (e.g. ABCB1 (MDR1), ABCC1 (MRP1)) has strong ATPase activity; these proteins are direct transport pumps. Here, the energy provided by ATP hydrolysis is used for the translocation of molecules across membranes. The other group of ABC transporters (e.g. ABCA1, ABCC7 (CFTR), ABCC8 (SUR1)) shows very low ATPase activity. Here it seems more likely, that these proteins act as a transport facilitator rather than an active transporter. Conformational changes due to ATP binding could be linked to regulatory processes. The ABC superfamily, which consists of currently 48 known human ABC transporters, is divided into seven subfamilies (ABCA to ABCG) by phylogenetic analysis. One group, the ABCA subfamily contains 12 full-size transporters (ABCA1 to ABCA10, ABCA12 and ABCA13), of which ABCA1, which regulates efflux of cholesterol from cholesterol rich cells, is in the focus of this work.

2.1.2 ABCA1 and Tangier Disease

In 1965 Fredrickson et al.[16] discovered a familial high-density lipoprotein deficiency in two siblings living on Tangier Island in Chesapeake Bay (Virginia, USA), which was named “Tangier Disease”. This autosomal codominant disorder is characterized (in the homozygous state) by the absence of HDL cholesterol from plasma, hepato-splenomegaly, peripheral neuropathy, and frequently premature coronary artery disease (CAD). In heterozygotes, HDL cholesterol levels are about half-normal.

Impaired cholesterol efflux from macrophages leads to the presence of foam cells throughout the body, which may explain the increased risk of coronary heart disease in several Tangier families. In 1999, the gene involved in this disease was identified.[17-19] People with Tangier Disease were homozygous or compound heterozygous for mutations in the gene ABC1 (later dubbed ABCA1). Although ABCA1 has already been discovered in the murine system in the mid-1990s, only little was known about its biological function until 1999.[20]

2.1.3 ABCA1 plays a key role in cellular cholesterol and phospholipid efflux

Many epidemiological studies have shown a reciprocal relationship between arteriosclerotic coronary heart disease and HDL levels. The prevailing opinion is, that HDL plays a pivotal role in reverse cholesterol transport, a process that delivers excess cholesterol from macrophages within the arterial wall to the liver for disposal. This efflux prevents the accumulation of cellular cholesteryl esters and foam cell formation. The cholesterol is carried in HDL back to the liver, where it is converted into bile acids and secreted into bile. In this process, called reverse cholesterol transport, the ABCA1 transporter plays a key role, since it facilitates the efflux of cholesterol and phospholipids to lipid-poor apolipoproteins. In Fig. 2.1, the metabolism of HDL and HDL precursors is illustrated. In liver and intestine and during extra hepatic degradation of chylomicrons, apo AI rich discoidal preβHDL particles are formed. The apo AI induces ABCA1-mediated efflux of phospholipids and cholesterol of cholesterol rich cells, to generate phospholipid rich HDL precursor particles. These are converted to regular HDL by different reactions, such as phospholipid transfer protein (PLTP) mediated uptake of triglycerides (TG) and phospholipid (PL) from TG- and PL-rich particles, and by influence of lecithin:cholesterol acyltransferase (LCAT). A second mechanism involves aqueous diffusion of cholesterol from the plasma membrane to HDL_3 particles along a concentration gradient continuously provided by LCAT that converts free cholesterol to cholesteryl ester (CE). Part of the mature HDL particles accumulate apo E and can then be uptaken by the liver via apo E- receptor. Alternatively, cholesteryl esters can be transferred from the HDL particles to apo B-100 rich lipoproteins via cholesteryl ester transfer protein (CETP). The formed apo B-100 rich LDL particles can then be uptaken by the liver via apo B-100 receptor. In addition, a selective uptake of cholesterol from CE-rich HDL particles mediated by scavenger receptor BI (SR-BI) has been proposed.[21, 22]

In addition, at least two more, ABCA1-independent pathways for efflux of cellular cholesterol are known: A significant basal cholesterol and phospholipid efflux has been found in monocytes from Tangier patients, which have a defective ABCA1 protein product and hence, this pathway is independent of ABCA1. Here, other members of the ABC transporter family might be involved, such as MDR1 and ABCG1.[23, 24]

And it has been shown, that cholesterol can be oxidized to 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid, of which at least 27-hydroxycholesterol can be secreted without a lipophilic acceptor.[25]

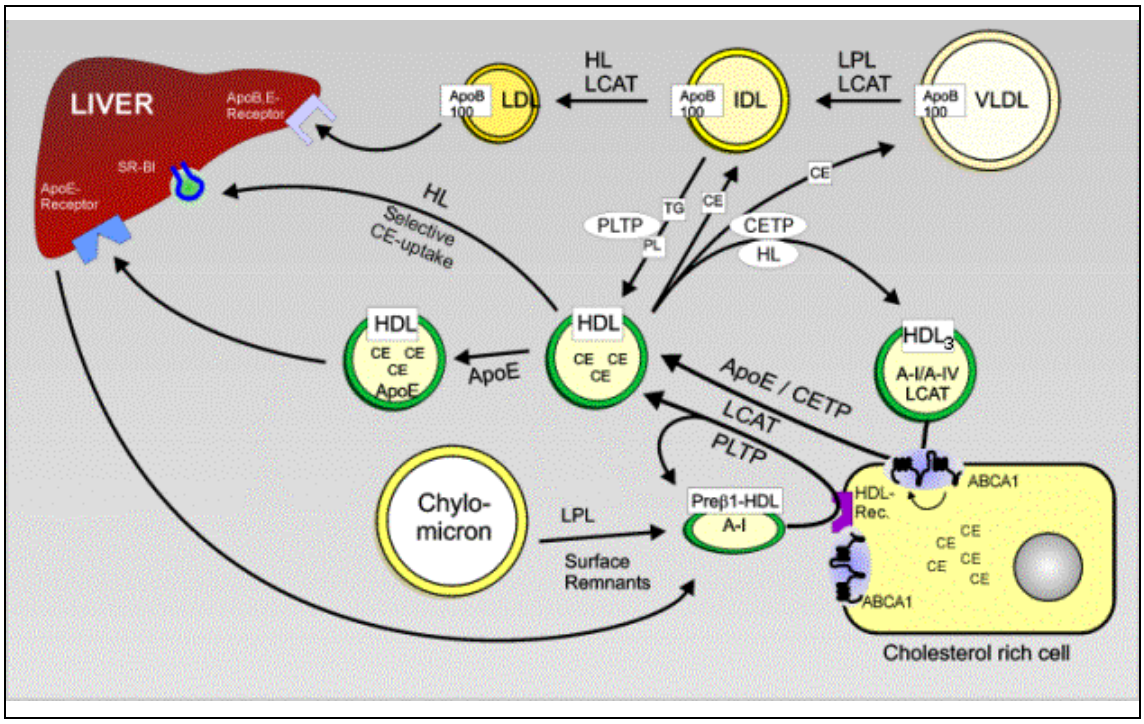

Fig. 2.1
Metabolism of HDL and HDL precursors [22]. ABCA1 facilitates efflux of cholesterol onto apo AI rich discoidal preβHDL and HDL_3, then transported to the liver for disposal.

2.1.4 ABCA1 defects

Disorders of HDL metabolism could result from mutations in genes along the metabolic pathway illustrated in Fig. 2.1, including apo AI, LCAT and ABCA1. Mutations in the ABCA1 gene have been identified as cause for high density lipoprotein (HDL) deficiency syndromes, including Tangier Disease. To clearly distinguish between Tangier Disease and other HDL deficiency syndromes, such as familial HDL deficiency syndrome, seems problematic. From a clinical point of view, typical Tangier Disease hallmarks include the recessive mode of inheritance, HDL deficiency, enlarged orange tonsils, hepatosplenomegaly, peripheral neuropathy and frequently premature onset of coronary heart disease. Familial HDL deficiency is diagnosed only on the basis of absence or reduced HDL levels. Since not all HDL deficient patients show typical signs of Tangier Disease, it has been proposed, that mutations in ABCA1 are diagnostic markers for incompletely expressed TD. However it could also be possible, that it depends on the type or location of the mutation in the ABCA1 gene, if a patient with a defective ABCA1 develops a typical symptom of TD such as splenomegaly or other HDL related diseases such as CAD.

The ABCA1 gene contains 50 exons and is located on chromosome 9 (9q31). Its open reading frame consists of 6,783 bp, coding a 2,261 amino acid protein product. The translation initiation site is located in exon 2. Since the identification of the ABCA1 gene being responsible for Tangier disease, several causative mutations have been identified in individuals with HDL deficiency, either in homozygous or heterozygous state. Individuals that are heterozygous for one of the causative mutations have reduced HDL-levels (40-45 %) and display some lipid aberrations, thus being at risk for cardiovascular disease.[15]

Up to now, 19 sequence variations upstream to the translation initiation site in exon 2 have been reported (see Tab. 7.2 and Fig. 7.2 of the appendix) and more than 60 sequence variations have been found in the coding region of ABCA1 (see Tab. 7.1 and Fig. 7.1 of the appendix). HDL deficiency syndrome related to mutations in ABCA1 has been reported in 38 patients from 28 kindreds so far. Most patients show either splenomegaly or coronary artery disease. Occurrence of both symptoms in the same patient is quite rare (see Tab. 7.3 and Fig. 7.3 of the appendix).

2.1.5 Regulation of ABCA1

With the ABCA1 gene playing a pivotal role in facilitating cholesterol efflux, it is of interest to determine how the ABCA1 gene itself is regulated. A number of factors and compounds have been identified to control or influence the expression of ABCA1, since it was initially identified as a sterol sensitive gene. In the promoter of the gene, several transcriptional control elements have been characterized (Fig. 2.2).

Tissue specific expression of ABCA1 is controlled by transcription factors Sp1 and Sp3, upstream stimulatory factor (USF) 1 and 2 and hepatic nuclear factor (HNF) 1α. Sp1, that binds to a GC-Box motif increases ABCA1 mRNA expression and enhances cellular cholesterol and phospholipid efflux, whereas Sp3 acts as a repressor and competes with Sp1 for binding at the –157 GnC motif. ABCA1 expression is regulated by cAMP and oxysterols that modulate binding of Sp1. It was shown that the conserved E-Box motif that binds USF 1/2 and HNF 1α has a silencing function, since mutagenesis of this motif increased ABCA1 expression.[26-35]

Furthermore, oxysterols and retinoids (e.g. 9-cis-retinoic acid) stimulate ABCA1 transcription through heterodimers of liver X receptor (LXR) and retinoic X receptor (RXR). The responsible control element was found to be an imperfect direct repeat of the nuclear receptor half-site TGACCT separated by four bases (DR4).[35-38]

In addition, the Zink finger protein ZNF 202 that maps to a hypoalphalipoproteinemia locus (11q23) has been reported to repress basal and induced expression of the ABCA1 gene and various other genes involved in lipid metabolism. It has been shown, that the amino terminal SCAN domain and the juxtaposed krüppel-associated box

(KRAB) domain of ZNF202 are required for its repressional activity. The SCAN domain mediates selective oligomerization with itself and other SCAN domain containing proteins such as ZNF191 and SCAN domain protein (SDP) 1. There is evidence that the KRAB domain-mediated transcriptional repression results from interference with the TATA box-dependent basal transcription machinery possibly in conjunction with the co-repressor KRAB associated protein (KAP) 1.[35, 39-44]

Additional putative control elements like binding sites for sex-determining region Y gene product (SRY), sex determining region Y-box (Sox) 5, nuclear factor (NF) kappa B and others are found upstream of the ZNF202 binding site. These are of unknown importance and have not yet been investigated further.

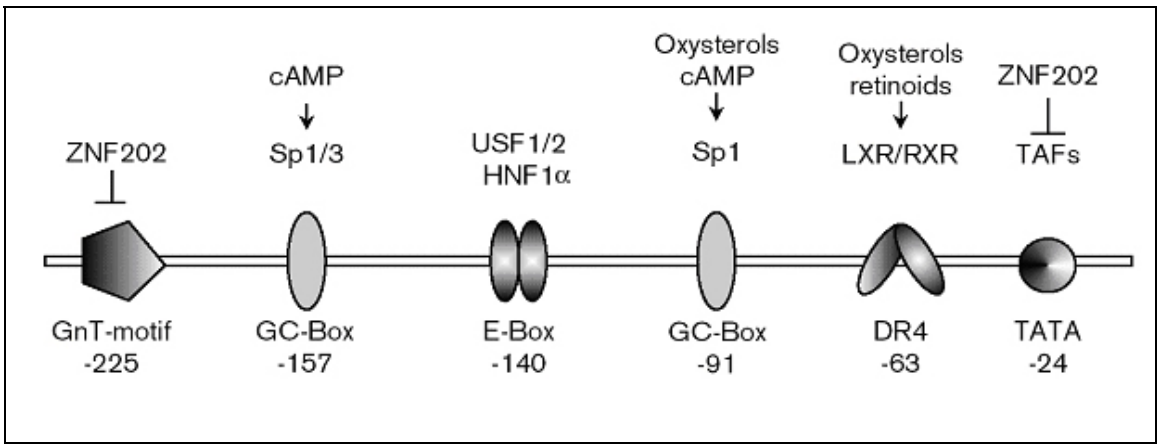

Fig. 2.2
The ABCA1 core promoter region contains transcriptional control elements for binding of ZNF202, liver X receptor (LXR) and retinoic X receptor (RXR). Furthermore, binding-sites for transcription factors Sp1 and Sp3, upstream stimulatory factor (USF) 1/2 and hepatic nuclear factor (HNF) 1α have been found, that regulate tissue specific expression of ABCA1.[35]

2.1.6 Important domains of ABCA1 and interacting proteins

As all other ABC-transporters, ABCA1 contains nucleotide binding domains and transmembrane domains. These domains are of importance to maintain ABCA1 function. Sequence variations in these regions should therefore be of increased significance, since they are very likely to affect ABCA1 function. Conversely, sequence variations that alter ABCA1 function could provide evidence for an important domain at this locus.

Since ABCA1 is believed to be no active transporter, because it has no measurable ATPase activity, it might act as a regulatory channel-forming protein like SUR1 and CFTR. Therefore, in analogy to CFTR, ABCA1 could bind SNARE proteins at its N-terminus to regulate the vesicular transport and the targeting of ABCA1 to the plasma membrane. Although, there is a strong conservation of several amino acid stretches in the N-terminal part of all ABCA transporters, which could indicate an essential function

of theses peptides, there is still no evidence to confirm this. However, like CFTR, ABCA1 contains a PDZ binding domain at its C-terminus and it has been shown, that this part of the protein interacts with β2-syntrophin.[45, 46]

In addition, a 23 aa long sequence rich in proline, glutamic acid, serine and threonine (PEST-sequence) has been found in the ABCA1 gene at codons 1284 – 1306 (coded in exons 27 and 28). It has been shown, that this sequence modulates the degradation of ABCA1 by calpain proteases.[47]

Furthermore the cell division cycle 42 (Cdc42) small GTP binding protein that controls cell polarity, cytoskeletal remodeling and vesicle transport was found associated with ABCA1 function and filopodia formation, but the mechanism and the domains involved in Cdc42 interaction with the ABCA1 pathway are not identified so far.[46, 48, 49]

2.2 Formats for SNP identification and analysis

In order to investigate the effects of SNPs in the ABCA1 gene, a suitable method has to be selected for the detection of these sequence variations. Today, numerous formats of wetware exist for preparation of allele distinguishable products. The various procedures can roughly be divided into hybridization techniques and enzymatic methods. This paragraph gives a brief overview of enabling techniques for SNP discrimination. For the development of high-throughput and multiplexing technology, an optimal wetware has to be selected.

2.2.1 Hybridization techniques

Hybridization techniques like the competitive hybridization (Fig. 2.3 A) usually involve the binding of complementary oligonucleotide sequences to the locus carrying the SNP query position. A problem with the detection of SNPs using complementary oligonucleotides is the similarity of their sequences. A difference of only one nucleotide will result in significant cross talk. Therefore, usually appropriate buffers, temperature adjustment or multiple washing steps are required to increase stringency. An alternative would be the use of specially modified oligonucleotides like the use of minor grove binders (especially used in TaqMan SNP analysis, see 2.3.3), which significantly increase the melting temperature of oligonucleotides. Hence, shorter oligonucleotides can be used, resulting in a higher match/mismatch ratio and so producing a more stringent annealing. In competitive hybridization, usually the unbound oligonucleotide or the decline of free oligonucleotide is detected by solid-phase binding (chip or bead-based technologies). For the detection, the oligonucleotide has to be labeled with fluorescent dyes or other appropriate tags.[50]

Another application is the use of melting curves with a single hybridization probe (Fig. 2.3 B). The probe binds at low temperatures to the desired position, whether there is a

mismatch or not. When steadily increasing the temperature of the reaction mixture, a miss-matched probe will melt off earlier than a probe bound with perfect match. Hence, the different alleles can be distinguished by their different melting points. The measurement of the melting curve can be done by a FRET (fluorescence resonance energy transfer) technique. An anchor probe, carrying a fluorescent donor dye and melting off significantly higher than the hybridization probe, is bound adjacent to the hybridization probe that carries a suitable fluorescent acceptor dye. As long as both probes are bound to the template DNA, FRET occurs, resulting in quenching of the fluorescence of the donor dye and emission of the acceptor dye. Continuously monitoring the fluorescence of the acceptor dye during the melting process will show a decline of the emission signal when the hybridization probe starts to melt off. The first negative derivative of the recorded fluorescence- temperature function will represent the melting point (50% dissociation) as peak. This format of wetware is especially used in LightCycler SNP analysis (see 2.3.2).[51]

Sophisticated hybridization probes like molecular beacons (Fig. 2.3 C) increase stringency of allelic discrimination. A molecular beacon is an oligonucleotide with the relevant sequence for hybridization and a self-complementary sequence at its 3'- and 5'-end, unrelated to the target sequence. For this reason, the unbound oligonucleotide forms a so-called hairpin or stem-loop structure, with the complementary target sequence in the loop and the self-complementary sequence in the stem. During hybridization, the probe will only form a hybrid with the target molecule, if the probe-target hybrid is more stable than the self-complementary stem structure. This not only increases stringency, a hybridization event to the target sequence can easily be monitored by applying a FRET technique. The 3'- and the 5'-end of the molecular beacon are both labeled, one end with a fluorescent moiety, the other one with an appropriate quenching moiety. When the oligonucleotide forms a hairpin structure, quencher and fluorophore are in close proximity and FRET takes place (the rate of energy transfer in FRET is indirect proportional to the sixth power of the distance between donor and acceptor). Upon hybridization, both arms of the probe gain a significant distance of each other and FRET can no longer occur, this causes the fluorescent moiety to emit light.[50, 52]

Scorpions are so-called self-priming probes. These are hybridization probes with a hairpin structure (like molecular beacons), which are attached via a spacer to a PCR primer. The spacer is necessary to cease elongation of the complementary strand during PCR beyond that point, to make sure that the hair-pin-part of the generated amplicon remains single-stranded. After denaturation of the double-stranded PCR product, the scorpion binds to the SNP query position if the detection sequence harbored in the loop part is exactly complementary to the target sequence (Fig. 2.3 D).

Like molecular beacons, stringency of hybridization is increased by the self-complementary parts of the probe and an appropriate fluorophore and quencher at both ends of the stem part can be used to monitor the hybridization event.[53]

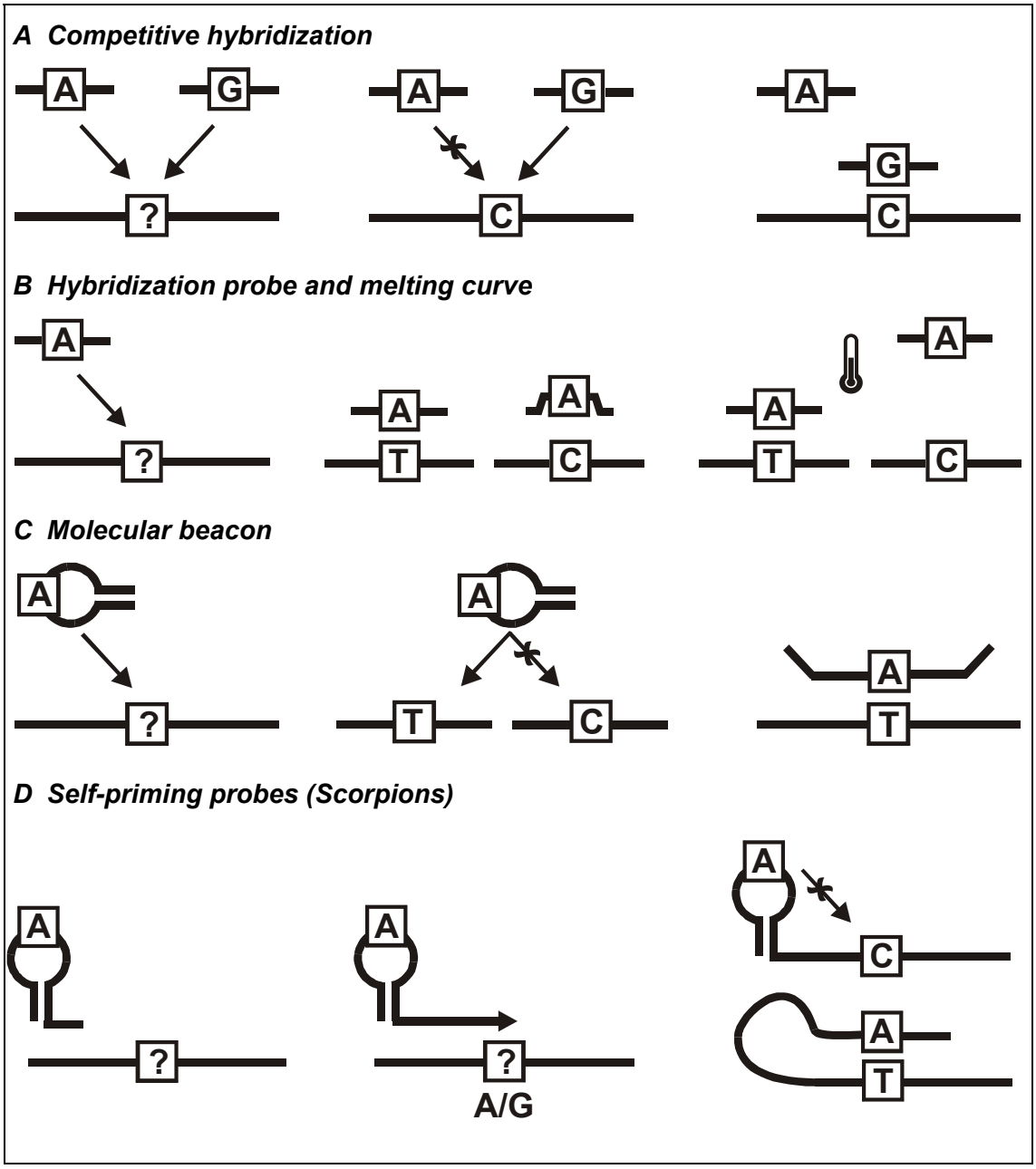

Fig. 2.3
Schematic representation of hybridization techniques for SNP discrimination.

2.2.2 Enzymatic methods

In PCR-RFLP (restriction fragment length polymorphism), subsequent to amplification of a relevant region by PCR, enzyme digestion is performed. Sequence recognizing restriction enzymes are used, that cut DNA only, if a specific sequence is found in it (Fig. 2.4 A). After enzyme digestion, the generated fragments are detected by agarose

gel electrophoresis. With a huge number of available enzymes, a large number of single nucleotide polymorphisms can be analyzed.[50, 54]

The Primer Extension reaction (Fig. 2.4 B) is a very common technique, used in multiple applications. Here, subsequent to PCR, only one primer is used that binds to the generated amplicon. This technique is often used in DNA sequencing. The relevant segment of DNA is amplified by PCR, then a primer (preferably one primer of the previous PCR) is extended by DNA polymerase. For this reaction, a mixture of unlabeled deoxynucleotide triphosphates (dNTPs) and labeled dideoxynucleotide triphosphates (ddNTPs) is used. The ddNTPs lack a hydroxyl group at the 3'-position, thus subsequent to incorporation, primer extension will terminate. Since the polymerase used cannot distinguish between dNTP and ddNTP, all possible lengths of fragments will be generated, each terminated with a ddNTP. By using a different label for each type of ddNTP, the reaction can be carried out in one approach; by using only one or two different labeled chain-terminators, up to four reactions have to be performed in parallel. An alternative to using labeled ddNTPs is the use of a labeled primer for extension. Then, four reactions have to be carried out in parallel, each with a different (unlabelled) ddNTP. The generated fragments are subjected to gel or capillary electrophoresis, aligning them by their size. Since the fragments carry a known base at their 3'-end (identified by the label), the targeted sequence can simply be assembled. In pyrosequencing, only one type of dNTP is added at a time. If the nucleotide is incorporated, pyrophosphate (PPi) is released, which can be detected by a multi-enzyme system producing luminescent light. Pyrosequencing can be used for sequencing of shorter fragments as well as for the detection of SNPs. In the latter case, the primer for extension is placed as close as possible to the SNP query position.[50]

The use of primers that bind upstream the SNP query position, with their 3'-end exactly one nucleotide before the SNP allow the extension of only one nucleotide, complementary to the SNP query position. This primer extension method is also called minisequencing. Here solely ddNTPs are used, to guarantee the elongation of the primer by a single base. The identification of the incorporated nucleotide can be determined by different (fluorescent) tags or by the presence or absence of a tag; this method is also known as template-directed dye-terminator incorporation (TDI) assay. Genetic Bit analysis is a method applying colorimetric detection of incorporated ddNTPs. Using MALDI-TOF mass spectrometry, the identity of the incorporated polymorphic nucleotide is detected at an m/z value specific to the added ddNTP. Fluorescence Polarization allows distinguishing between incorporated and non-incorporated fluorescently labeled nucleotides.[50]

In allele specific PCR, one of the used primers has a polymorphic base at or very close to its 3' end. The formation or the rate of formation of a PCR product then depends on

the allele present in the template DNA since only a so-called allele specific oligonucleotide (ASO) with a matched 3'-residue will function as primer. Usually, one reaction is run for each allele. The successful formation of PCR product can be detected by gel electrophoresis (amplification refractory mutation system, ARMS) or by real time PCR (kinetic PCR) using intercalating dyes such as Ethidium bromide or SYBR Green.[50]

Allele specific primer extension (ASPE, Fig. 2.4 C), combines primer extension and allele specific PCR. Primers carry the polymorphic base at their 3'-residue and are only extended, when they match the SNP query position. Compared to minisequencing, no differently labeled ddNTPs are needed. Only one labeled dNTP (usually dCTP) is used in this reaction, which is incorporated multiple times upon extension, hence signal intensity is increased. This reaction has been used in solid phase technologies, with ASOs, carrying a 5' dangling end used for immobilization of the generated product to the complementary sequence bound to solid support.[55]

In the oligonucleotide ligation assay (OLA, Fig. 2.4 D), two adjacent hybridized oligonucleotides are enzymatically ligated to each other using DNA ligase. This reaction only takes place, when the nucleotides next to the ligation position are fully complementary. By this means, one of the used oligonucleotide must carry the allelic base at the very 5'- or 3'-end. The 3'-end is even more discriminatory than the 5'-end, but both procedures work well. This method has been used in chip technology and bead-based analysis platforms, using one fluorescently labeled reporter oligonucleotide that can be ligated to another oligonucleotide, carrying a 5'-dangling end, which can bind to the chip or bead (zip code). By attaching a FRET donor dye to one oligonucleotide and an appropriate acceptor dye to the adjacent oligonucleotide, a homogeneous assay can be established, which is carried out on a real time PCR machine. This method is also referred to as dye-labeled oligonucleotide ligation (DOL) assay.[50]

OLA has also been used in combination with rolling circle amplification (RCA, also called rolling circle replication RCR). Here, circularizing oligonucleotide probes (padlock probes) are used that anneal with their 5' and 3' end to the target SNP forming an open circle. With the polymorphic base usually located at the 3' end of the open circle probe, ring closure by oligonucleotide ligation does only occur upon perfect complement of the hybridizing ends of the probe. After ring closure, a primer extension reaction is performed, using a primer that binds to the oligonucleotide probe and a polymerase with strand displacement activity. If the probe has been circularized, a long single-stranded DNA containing multiple tandem repeats complementary to the original circle sequence is formed. A large number of labeled dNTPs can be incorporated

during RCR, resulting in a powerful signal amplification. This allows SNP discrimination directly from genomic DNA, no amplification step is required in advance. [54, 56-60]

The 5‘-exonuclease assay (Fig. 2.4 E), also called TaqMan assay, is performed using two detection probes, each recognizing a specific allele. For allelic discrimination, the oligonucleotides are usually modified with a minor-groove binder (MGB) at the 3’-end. The MGB increases the melting temperature of the probe thus allowing a more stringent annealing. During PCR, a probe with a perfect match will be cut into pieces by the 5’-3’ exonuclease activity of the Taq polymerase, while a mismatched probe will be displaced. For detection, each probe is labeled with a fluorophore on the 5’ end and an appropriate quencher attached to a MGB on the 3’ end. The NFQ quenches the fluorescence of the fluorophore as long as both are attached to the same oligo-nucleotide. Degradation of the probe by the 5’-3’-exonuclease activity releases the fluorophore from the vicinity of the quencher, thus it will emit light upon excitation. So after PCR, simply the end-point fluorescence intensity needs to be measured.[50]

In the invader assay (Fig. 2.4 F), a flap endonuclease (FEN) is used for allelic discrimination of SNPs. This DNA repair enzyme recognizes and cleaves a redundant 5’-portion (flap) of the downstream segment, when two overlapping DNA segments are bound to the complementary sequence. In the assay, two probes are used, an invader oligonucleotide hybridizing with the targeted complementary polymorphic base at the 3’-end and a signal oligonucleotide hybridizing downstream to the SNP and overlapping the invader oligonucleotide. The 5’-flap of the signal probe is usually not complementary to the target sequence and will be cleaved off by FEN, if the last base of the invader oligonucleotide does match the polymorphic base. Otherwise, a structure is formed that cannot be recognized by FEN and the signal probe will not be cleaved. Invader assays have been shown to function without a previous PCR and linear amplification of the cleaved product works without temperature cycling. In a so-called invader squared assay, the cleaved off flap of a primary signal probe serves as invader oligonucleotide in a secondary invader reaction. This two-step reaction squares the amount of amplification of the cleaved product. The invader assay has been shown to function with MALDI-TOF, but fluorescent-based techniques, like FRET, should also work.[50, 54, 61, 62]

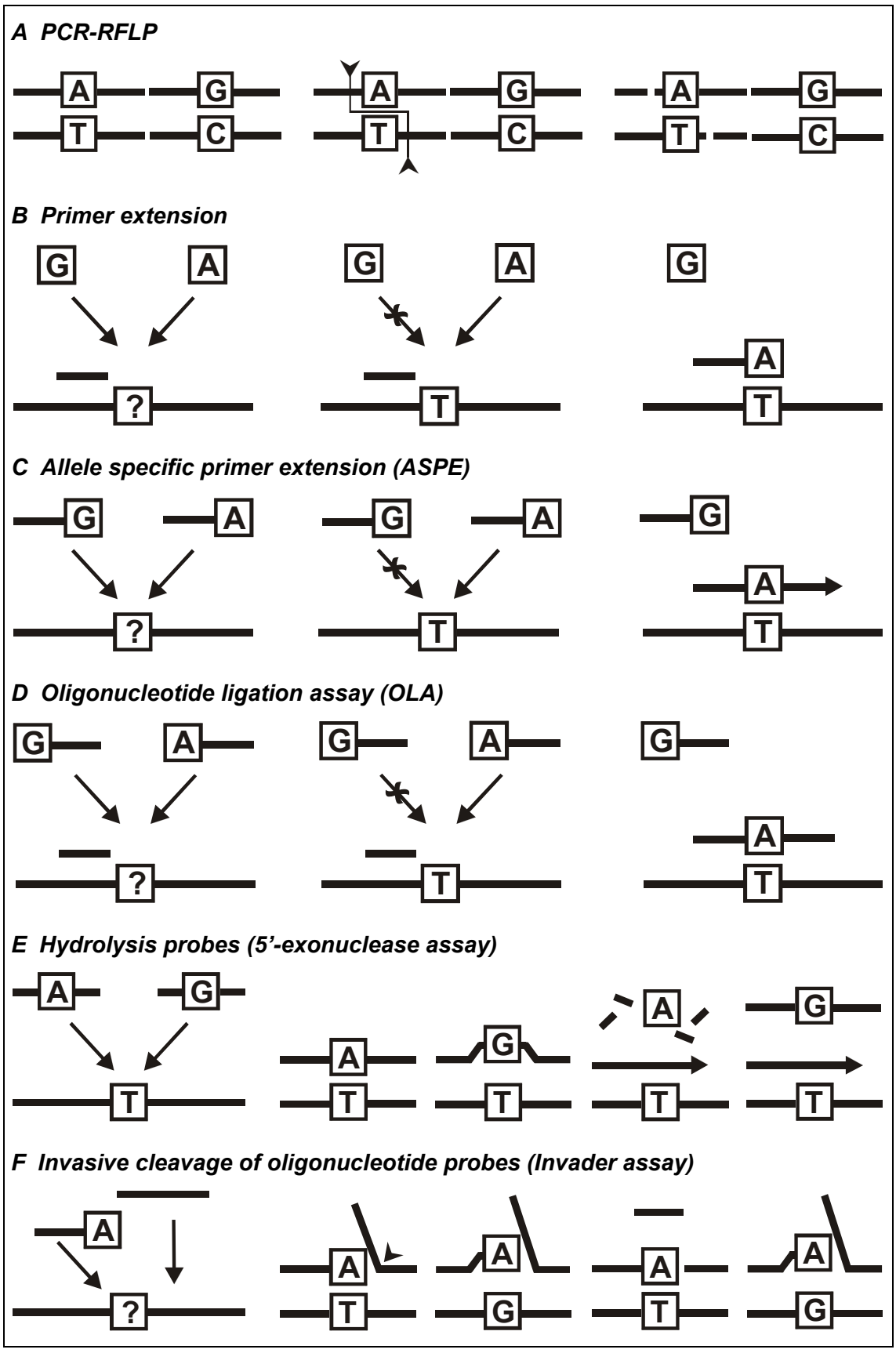

Fig. 2.4
Schematic representation of enzymatic techniques for SNP discrimination.

2.3 Instrumentation for SNP analysis

Various platforms for detection of discriminated SNP products exist. Most novel platforms are fluorescent based, since fluorescence is a very sensitive and versatile technique. In this chapter only platforms that have been used for discovery, development and evaluation of SNP analysis for the ABCA1 gene will be described: capillary sequencer, LightCycler and TaqMan. Other well established platforms at this time are fluorescence polarization, pyrosequencing, and MALDI-TOF, however these platforms will not be discussed here.

2.3.1 Capillary sequencer

DNA sequencing is an essential technique for the identification of novel sequence variations, including SNPs and for the detection of multiple sequence variations in highly polymorphic regions. Although genotyping of single SNPs can be performed by DNA sequencing, it is usually not done if alternative, more cost-effective techniques are available. With the ABI Prism 3100 sequencer, the most commonly used method for DNA sequencing is applied, the Sanger dideoxy sequencing. Here, chain-terminating dideoxy nucleotide triphosphates are used in the sequencing reaction. The ddNTPs carry a fluorescent tag, which identifies the incorporated nucleotide by the wavelength of the emitted light. Usually, fluorescein dyes like FAM, JOE, ROX, etc. were used before they recently have been replaced by BigDyes. BigDyes are intra-molecular FRET dyes (energy transfer-dyes), which consist of a derivative of a 5-carboxy-dichlororhodamine acceptor dye and a 5- or 6-carboxyisomer of 4'-aminomethyl-fluorescein as donor dye. A rigid linker (4-aminomethylbenzoic acid) is used to separate both residues. The BigDye is connected via a proparagyl ethoxyamino linker to the dideoxy nucleotide triphosphate. Fig. 2.5 shows the structure of the ddTTP-BigDye terminator as an example.[63]

Since dNTPs or ddNTPs are incorporated by the DNA polymerase all possible lengths of DNA fragments are produced. In capillary electrophoresis, the generated fragments travel though a capillary filled with a polymer, where they are separated by their size. In the optical window of the capillary, near the anode, the fragments traveling by, are excited by a 488 nm Argon laser. The emitted fluorescence of the Big Dye terminators is detected by means of a CCD camera. A set of dye terminators is required, capable of being excited at 488 nm and showing sufficient spectral separation of emission light (Fig. 2.6).[64]

With the ABI Prism 3100 capillary sequencer, 16 samples can be processed in approximately one hour.

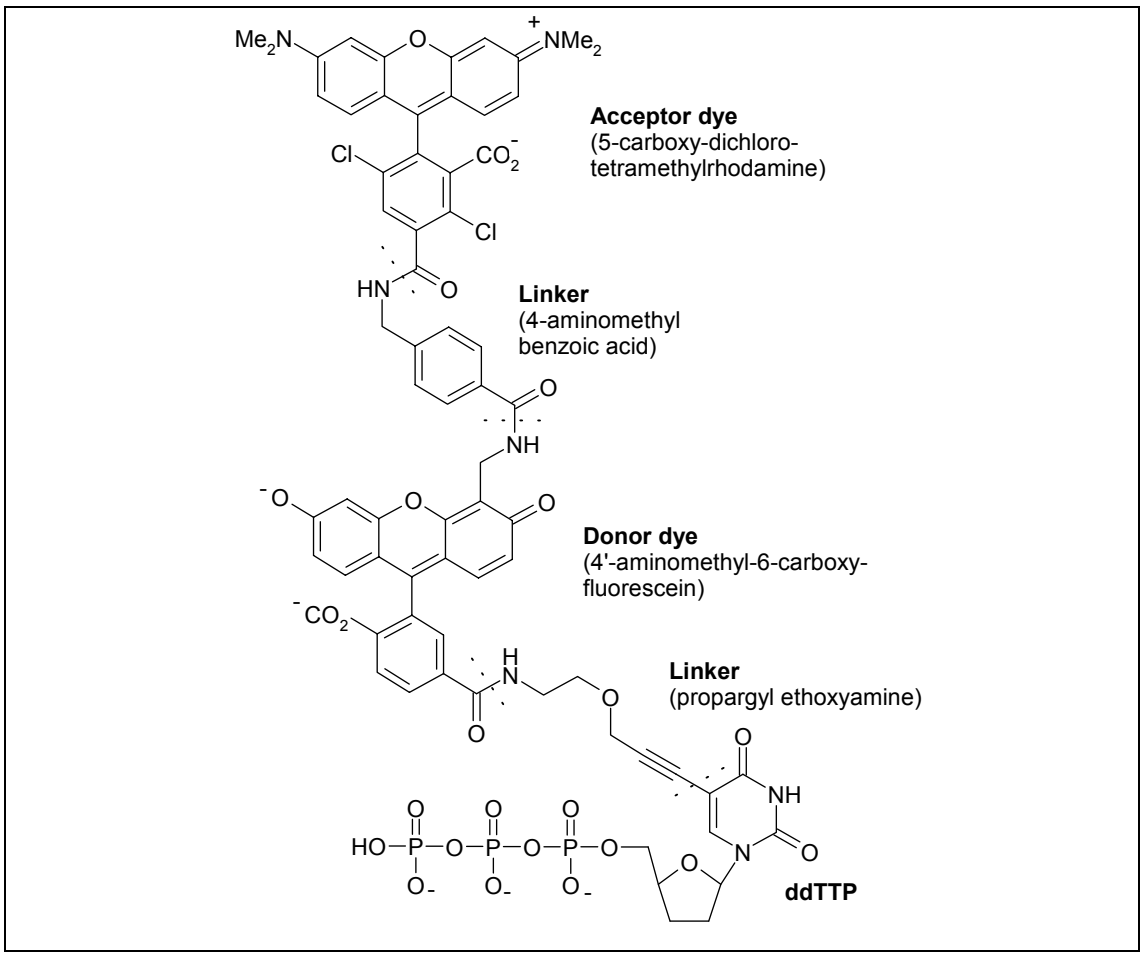

Fig. 2.5
Structure of BigDye-ddTTP. The intra-molecular FRET dye consist of a 5-carboxy-dichlororhodamine acceptor dye and a 6-carboxyisomer of 4'-aminomethylfluorescein as donor dye which are connected via the rigid linker 4-aminomethylbenzoic acid. The BigDye is bound via a proparagyl ethoxyamino linker to the dideoxy nucleotide triphosphate.[63]

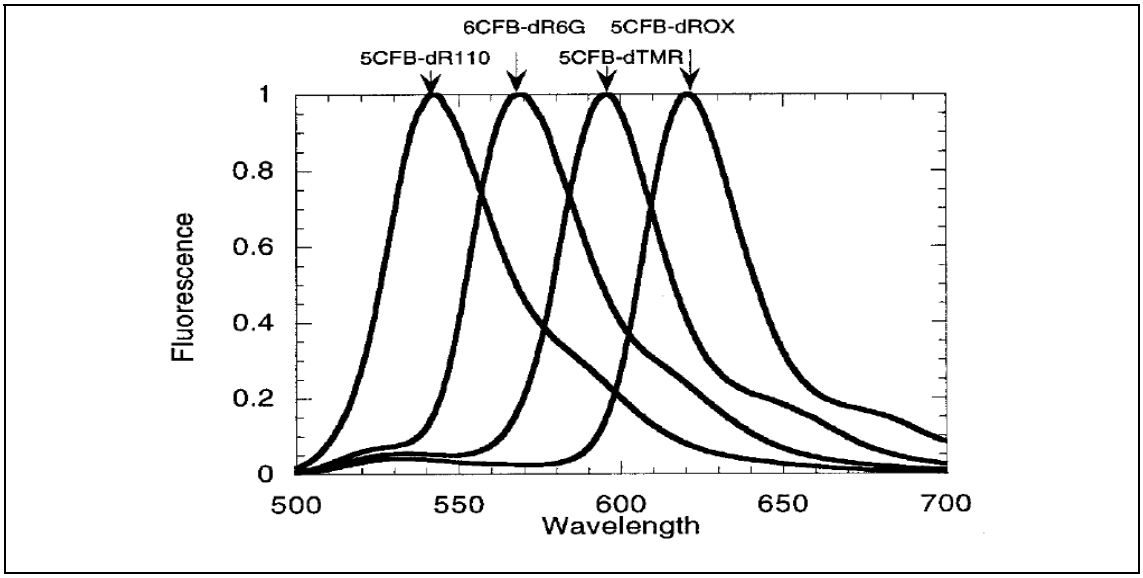

Fig. 2.6
Normalized emission spectra of BigDye terminator dyes (excitation: 488 nm).[64]

2.3.2 Light Cycler

The LightCycler (Roche Diagnostics, Basel, Switzerland) is a microvolume multisample fluorimeter with rapid temperature control. The outstanding speed of the thermal cycler (30 temperature cycles in 15-20 minutes) is achieved by forced air heating using a chamber fan. Small size samples (10-20 µl) are processed in thin glass capillaries that allow rapid heat transfer due to a high surface to volume ratio. The capillaries are made of clear borosilicate glass, which is ideal for fluorescence analysis, due to its optical properties. Furthermore, a capillary conducts emitted light to its tip in a way, which is comparable to fiber optics, producing an intense fluorescent signal at the tip. The cylindrical sample holder (carousel) carries up to 32 capillaries, which are positioned to the detection spot by a stepper motor quickly rotating the carousel. Fast fluorescence optics were adopted from flow cytometry. The light beam, originating from a blue light diode (480 nm), is focused on the capillary tip from below. The emitted fluorescent light is detected from the capillary tip by means of three photodiodes with appropriate filters (F1: 530 nm, F2: 640 nm and F3: 710 nm).[65, 66]

Due to patent situation and for historical reasons, SNP detection using the LightCycler technology is usually based on hybridization probes applying fluorescence resonance energy transfer (FRET). Other techniques like hydrolysis probes, molecular beacons and others can also be performed when using appropriate fluorescent tags, but they are less well established. As a donor dye for FRET, Fluorescein can be used, emitting in the F1 channel. LightCycler Red 640 and LightCycler Red 705 are appropriate acceptor dyes for Fluorescein, emitting in channel F2 and F3, respectively (Fig. 2.7).

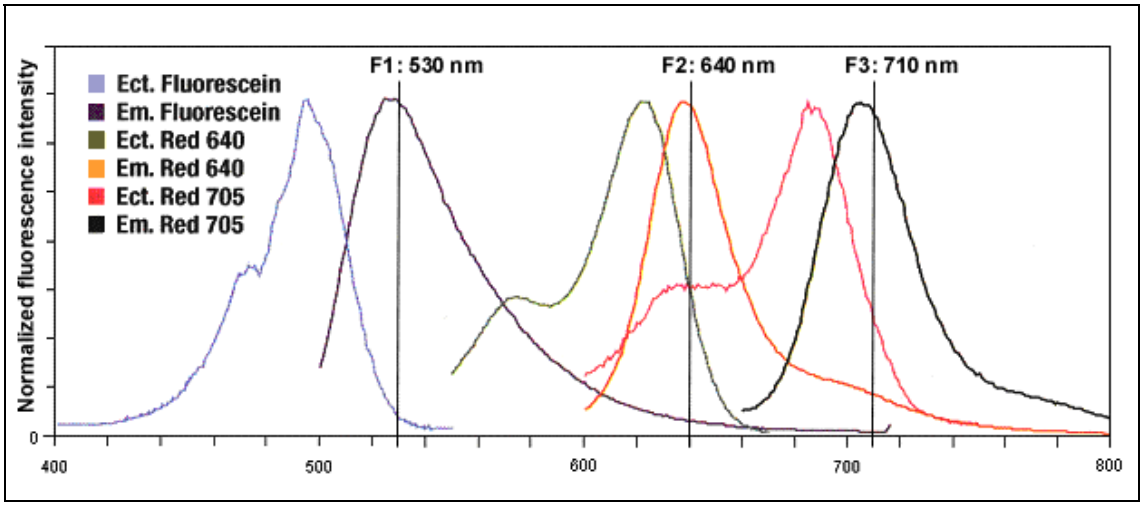

Fig. 2.7
Excitation and emission spectra of LightCycler dye set. As a donor dye for FRET, Fluorescein is used, emitting in the F1 channel (530 nm). LightCycler Red 640 and LightCycler Red 705 are appropriate acceptor dyes for Fluorescein, emitting in channel F2 (640 nm) and F3 (710 nm), respectively.

Other FRET dyes that match the integrated filter set can also be used. One example is Fluorescein together with Cy5 and Cy5.5 instead of LightCycler Red 640 and LightCycler Red 705, respectively.

For SNP analysis with the LightCycler, the DNA fragment of interest (harboring the SNP) is amplified using standard PCR primers under optimized conditions. Two sequence-specific oligonucleotide probes are used for the detection of the amplicon. The detection or sensor probe hybridizes to the SNP query position (marked with a black dot in Fig. 2.8) and carries a FRET acceptor dye (LightCycler Red 640 or LightCycler Red 705) at its 5' end. The anchor probe is coupled with a FITC derivative of Fluorescein at its 3'-end and binds upstream of the detection probe, leaving a gap of 1-5 nucleotides between the two probes. To prevent the sensor probe from extension by Taq polymerase, it must be phosphorylated at the 3' position. The anchor probe carries a fluorophore at its 3'-end and though cannot be extended. Only if hybridized to the amplicon, the two probes are in such close proximity, that FRET can occur and hence emission light can be detected in channel F2 or F3, respectively. Although the probe set can be used to monitor the emergence of PCR product during the annealing steps, which is of relevance when expression levels of mRNAs or copies of genomes are analyzed, its intended purpose is the allelic discrimination during melting curve analysis subsequent to the last PCR cycle. The reaction mixture is first denatured at 95°C and cooled to below 45°C, then the temperature in the thermal chamber is slowly raised (0.1-0.2°C/sec) and fluorescence is continuously monitored. The melting curve analysis allows the determination of the melting point of the probe-target hybrid. The detection probe spans the polymorphic site, if there is a mismatch or not. But a mismatch will destabilize the hybrid and the probe melts off at a lower temperature than with a perfect match. Fig. 2.9 illustrates this principle. The melting curve is plotted as temperature against fluorescence intensity (FI). At the melting point of the probe-target hybrid, per definition, 50% of the probe is annealed to the target. This is represented by the turning point of the melting curve. For better visualization, the first negative derivative of the melting curve (dFI/dT) is calculated, which shows a maximum at the melting point. The determination of the melting point allows the assignment of the corresponding genotype and assessment whether the proband is homozygous or heterozygous (Fig. 2.9)

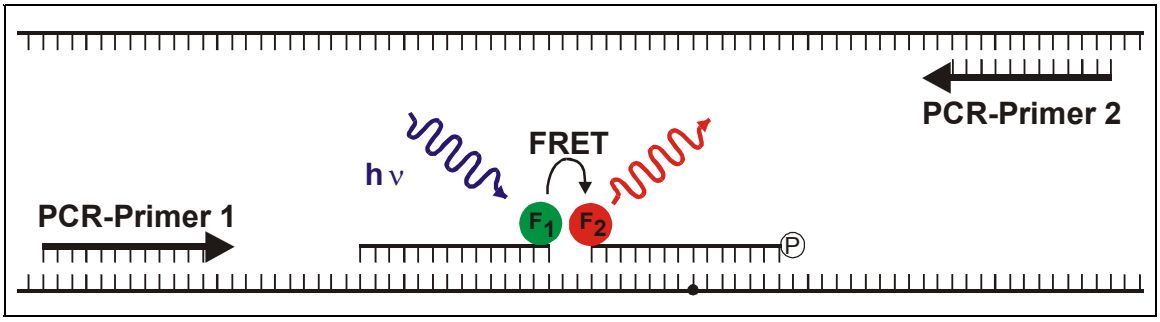

Fig. 2.8
The principle of FRET probes. The detection or sensor probe hybridizes to the SNP query position (marked with a black dot) and carries FRET acceptor dye F_2 at its 5' end. The anchor probe is coupled with donor dye F_1 at its 3'-end and binds upstream of the detection probe, leaving a gap of 1-5 nucleotides between the two probes. To prevent the sensor probe from extension by Taq polymerase, it is phosphorylated at the 3' position. Only if hybridized to the amplicon, the two probes are in such close proximity, that FRET can occur and hence emission light of F_2 can be detected.

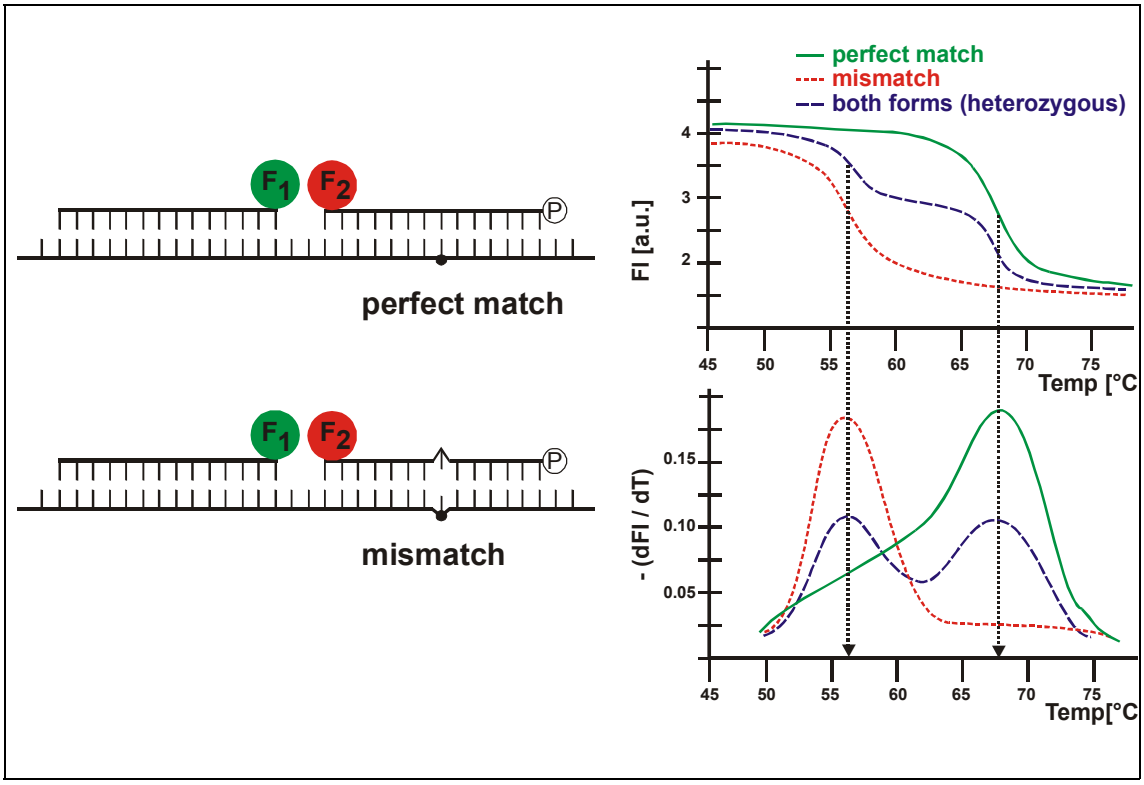

Fig. 2.9
The melting curve analysis allows the determination of the melting point of the probe-target hybrid. A mismatch will destabilize the hybrid and the probe melts off at a lower temperature than with a perfect match. The melting curve is plotted as temperature against fluorescence intensity (Fl). The first negative derivative of the melting curve (dFl/dT) is calculated, which shows a maximum at the melting point. The determination of the melting point allows the assignment of the corresponding genotype and assessment whether the proband is homozygous or heterozygous.

2.3.3 TaqMan

Although the name “TaqMan” refers to the 5’ nuclease assay (see 2.2.2) and is a registered Trademark of Roche Molecular Systems, it is often used – colloquial speaking – for analysis platforms from Applied Biosystems (Foster City, CA, USA) of type “ABI Prism Sequence Detection System”, which are commonly used with the 5’ nuclease assay. Among several different types of sequence detection systems, the ABI Prism 7900HT is especially designed for high-throughput analysis and was used for this work.

The 7900HT is a standard peltier-based thermal cycling system with a fluorescence excitation and detection unit. An Argon 488 nm laser is used as excitation source. The emission is detected by a CCD camera between 500 and 660 nm continuously, with a resolution of 5 nm, this allows multi-color applications. The movable dual-axis optical head of the system moves quickly over the 384-well microtiter-plate in the thermal cycler. The microtiter-plate is sealed with an optical cover slide and covered with a heated lid with a hole over each well. From above, the optical head pipes laser light into the samples and collects emission light simultaneously of 8 wells using fiber optics. After the data have been recorded, a virtual filter is set by the software to isolate the correct wavelength for the used fluorescent dye. The system has to be calibrated in advance, by recording the emission spectrum of each fluorescent dye to be used. Usually dyes from Applied Biosystems, especially the aforementioned fluorescein dyes are used (Fig. 2.10). The PCR master mix must contain an amount of ROX dye for normalization of all other fluorescence signals.

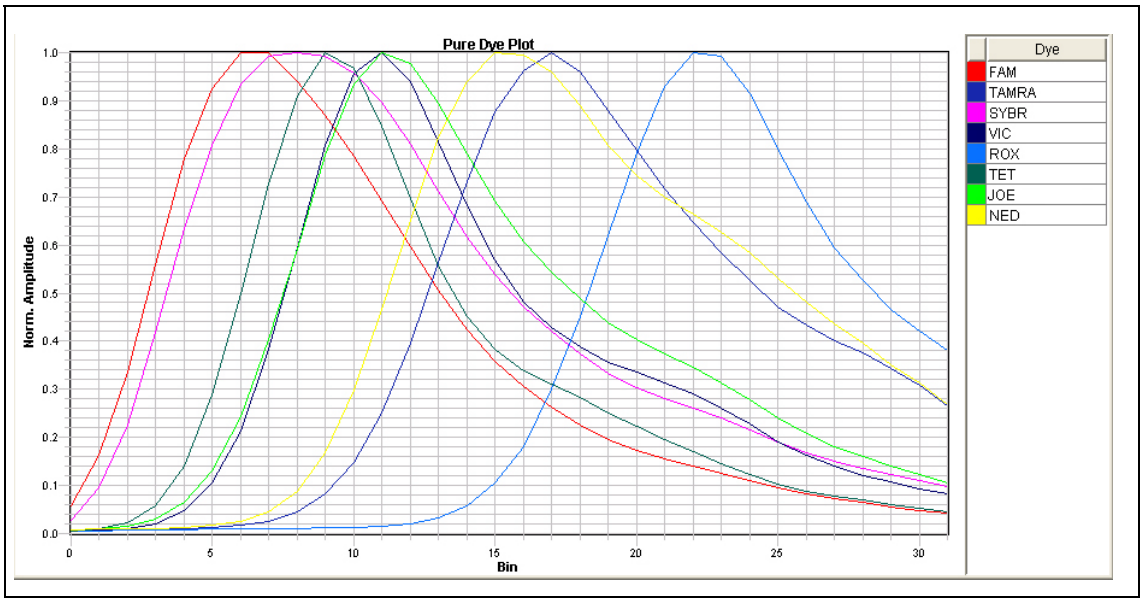

Fig. 2.10
Recorded (normalized) emission spectra of pure dyes between 500 and 660 nm. The spectrograph records one data point every 5 nm.

The TaqMan 7900HT is equipped with an integrated bar code scanner and a custom Zymark Twister, that can hold up to 84 microtiter plates, is used as an automatic plate loader for continuous plate loading without user intervention. In this configuration, the TaqMan can measure 26 microtiter-plates per hour, which corresponds to around 10,000 samples, if additional external thermal cyclers are used. The outstanding speed can be explained by the fact, that for the analysis of SNPs it is not necessary to continuously monitor fluorescence. Measurement of end-point fluorescence (subsequent to PCR) is sufficient and hence, the PCR reaction can be carried out in standard thermocyclers, without fluorescence unit.

As described previously (see 2.2.2), the 5' nuclease assay involves a probe with a fluorophore attached to its 5' end, and a suitable quencher at the 3' moiety. During PCR, a mismatched probe is displaced, while a perfect-matched probe will be degraded by the 5'-3' exonuclease activity of Taq polymerase, releasing the fluorophore from the quencher and hence producing fluorescent light (Fig. 2.11).

One probe for each allele is required. Usually, the two fluorescent dyes VIC and FAM are used as fluorophores for the TaqMan probes, together with a non-fluorogenic quencher connected to a minor groove binder molecule. The chemical structure of VIC, the non-fluorogenic quencher and the minor groove binder are a trade secret of Applied Biosystems. The minor groove binder is necessary to obtain a good discrimination of matched and mismatched probes. A minor groove binder (MGB) is a crescent shaped molecule, binding into the minor groove of double-stranded DNA and hence increasing stability of the duplex-structure. As an example, the MGB $CDPI_3$, a dihydropyrroloindole tripeptide is illustrated in Fig. 2.12, which can be attached to the 3'-residue of oligonucleotides and then binds into the minor groove of the formed double-helix structure and hence increases its stability (Fig. 2.13). A 12-mer oligonucleotide then has the same melting temperature (T_m) of 65°C as an unmodified 27-mer. The 12-mer probe had a 20°C difference in T_m between matched and mismatched duplex, which is very advantageous for single nucleotide discrimination. The use of short MGB probes compared to standard oligonucleotide probes provides hence an increased specificity due to a higher mismatch-to-match-ratio and an increased sensitivity due to more efficient quenching caused by a lower separation distance between fluorophore and quencher.[67-69]

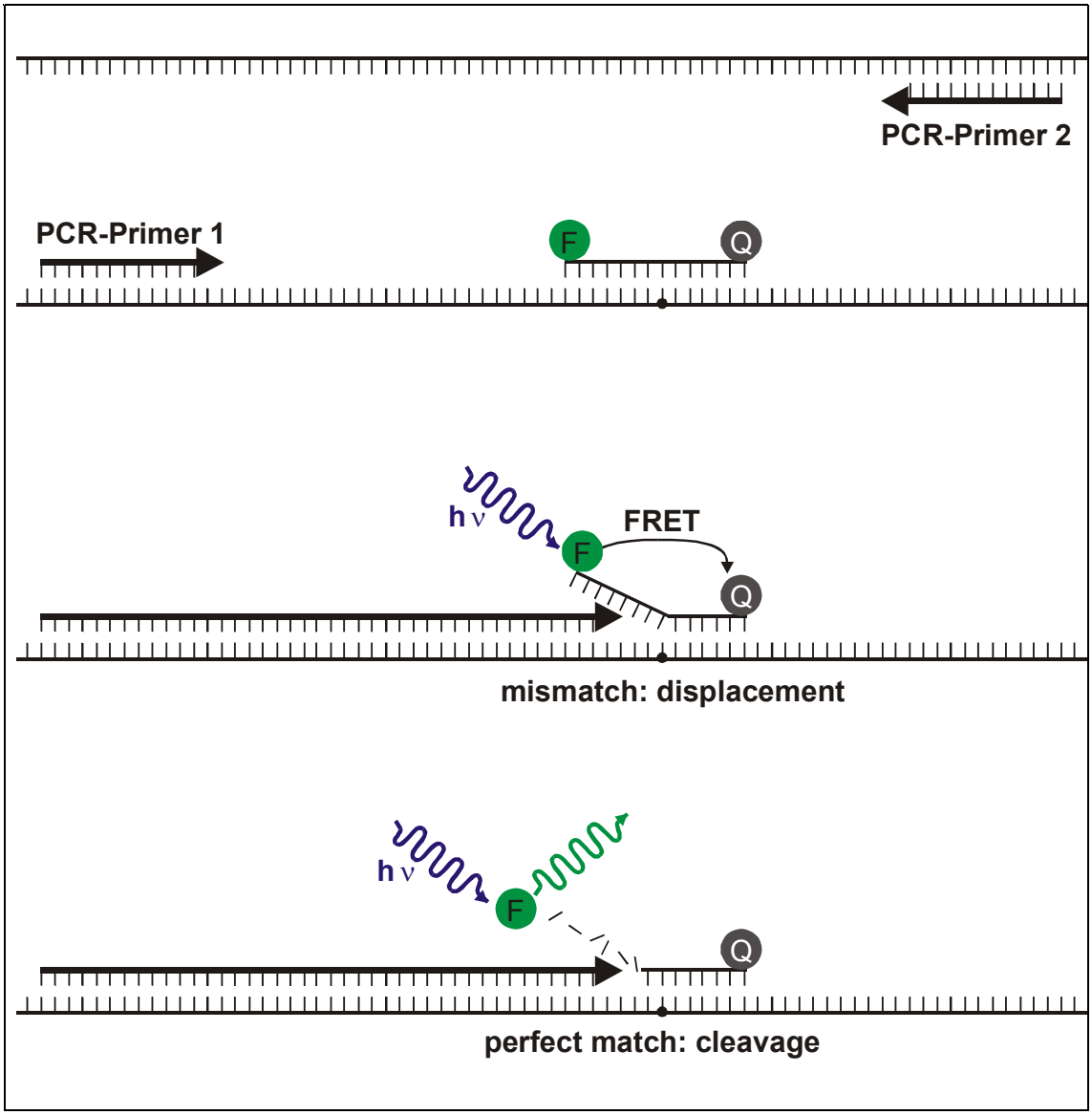

Fig. 2.11
The TaqMan assay involves a probe with a fluorophore attached to its 5' end, and a suitable quencher at the 3' moiety. During PCR, a mismatched probe is displaced, while a perfect-matched probe will be degraded by the 5'-3' exonuclease activity of Taq polymerase, releasing the fluorophore from the quencher and hence producing fluorescent light.

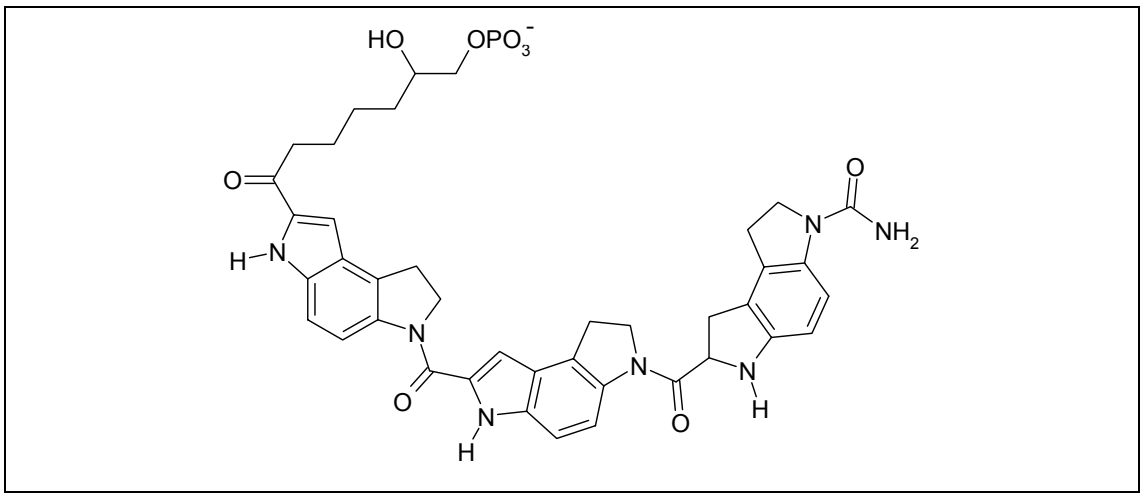

Fig. 2.12
Chemical structure of minor groove binder $CDPI_3$ (tri-peptide of N-3-carbamoyl-1,2-dihydo-3H-pyrrolo[3,2-e]indole-7-carboxylate) with a phosphate linker.[67]

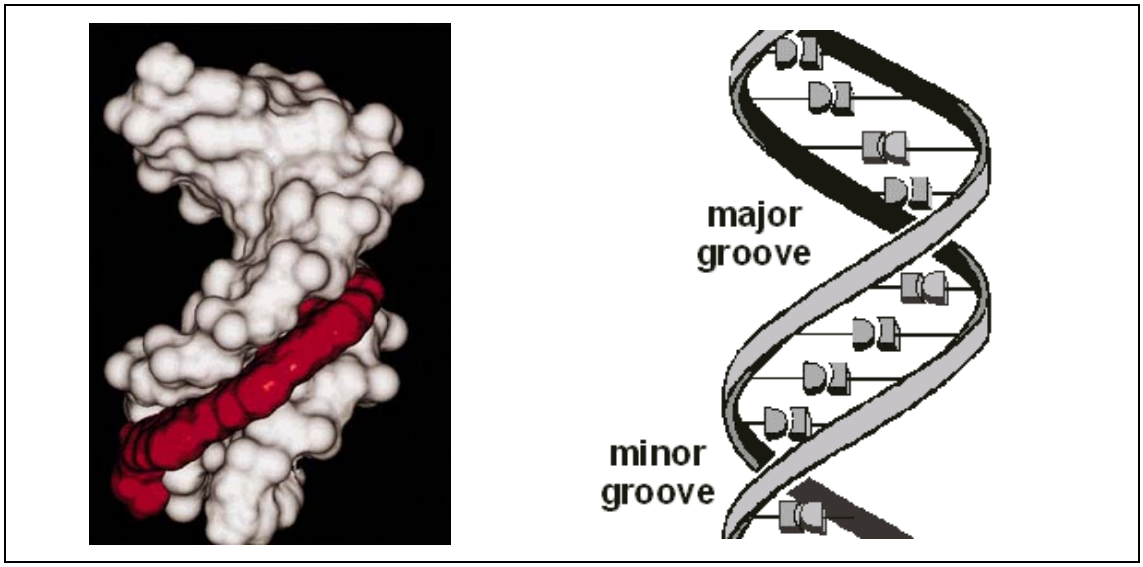

Fig. 2.13
Left: Connoly surface representation of calculated structure of a double-stranded oligonucleotide decamer with attached (red) $CDPI_3$.[69] Right: Schematic illustration of the double helix structure, showing minor and major groove.

2.4 Bead-based multiplex analytical platforms

In genomics, test platforms which allow to characterize a single biological sample regarding multiple analytes simultaneously have recently been broadly introduced in the format of high density DNA arrays, which mainly serve sequence verification, gene expression monitoring and detection of sequence variations.

An important disadvantage of arrays is the low flexibility in the selection of combinations of analytes on pre-manufactured chips. These chips typically cover a large number of parameters to suit different individual needs and hence find a ready market. This allows mass production and makes these chips affordable. But reducing the number of parameters to necessary analytes only can also cut analytical costs. Here, bead-based assays provide a promising and cost-effective alternative, since distinct bead species can be mixed individually to target multiple analytes in a single sample and then analyzed simultaneously. This saves reagent costs and reduces sample volumes. Less sophisticated instrumentation and hence lower investment costs for bead-based platforms are another important fact.

The first diagnostic application of beads was the latex agglutination test (LAT). It was invented in 1956 by SINGER and PLOTZ [70, 71], who introduced a rheumatoid factor agglutination test. With LATs, antibodies and high-molecular-weight antigens can be detected. Due to the fact that antibodies are not monovalent, high aggregates of antigen-coated beads and free antibody or antibody-coated beads and free antigen form, causing the highly disperse suspension of beads to form clumps. Since its invention, the LAT grew more and more popular since it required no sophisticated instrumentation, was rapid and required no extensively trained personnel. Very soon these beads were used as carriers for ELISA or FLISA and also introduced in flow cytometry.

In order to be used in flow cytometry, beads have to be homogeneous microspheres or microparticles, which are used as carriers for biomolecules in tests or assays. Beads made of polystyrene and other synthetic polymers are also referred to as "latex" particles, since they can be regarded as a dispersion of synthetic polymer. They are available in sizes ranging from 0.4 µm to about 200 µm. Like cells, beads are analyzed by flow cytometry, when the particles pass one by one through a light beam. Forward and side scatter of the light is measured and fluorescent dyes attached to the beads can be excited and their emission can be detected. Several hundreds of single beads are measured, giving a mean signal with high precision. A very important property to discriminate single beads from aggregates and debris is that they all have the same light scattering properties. This can only be achieved by using so-called uniform latex particles, having the same shape and size.

2.4.1 Synthesis, dying and coating of beads

The polymerization of emulsions of styrene for instance results in the creation of microspheres in multiple sizes. For the synthesis of particles with the same size, sophisticated methods like swollen emulsion polymerization or seed emulsion polymerization have been developed.[72] Swollen emulsion polymerization involves the use of an emulsifying agent like potassium laureate or sodium dodecyl sulfate, which form micelles of identical size in water. Styrene is added, which travels into the lipophilic interior of the micelles and is polymerized upon addition of a free radical initiation reagent. This method allows the production of particles up to 3 µm in diameter. To obtain larger particles, adding styrene to an aqueous suspension of microspheres can swell small particles. This process is called seed emulsion polymerization.[73]

In flow cytometry fluorescence intensity and spectral diversity are used to distinguish between several species. Hence, particles could be impregnated with fluorophore to generate spectrally distinct populations. Most beads are made of polystyrene or are at least copolymers of polystyrene; there are also silica beads, superparamagnetic beads with a magnetite core and beads made of polymethylmethacrylate and several other materials, but polystyrene gives beads the property of swelling in organic solvent, which is an important condition for dying the particles. Upon the addition of lipophilic solvent to an aqueous suspension of polystyrene beads, the lipophilic particles begin to swell by taking up the organic solvent. Lipophilic dyes dissolved in the organic solvent will by this way be transported into the interior of the particles. Azeotropic distillation or multiple washing steps will remove the organic solvent, causing the particles to shrink to their original size and trapping the dye in the interior of the bead.

In order to carry out assays with beads, they have to be “sensitized” by attaching biological ligands to their surface. This process is called coating and can be done either by passive adsorption or covalent coupling. Passive adsorption is due to hydrophobic attractions between the polymeric surface and hydrophobic parts of the ligand. Although passive adsorption is a very simple technique, it does not guarantee binding of the ligand in its most biologically active orientation (antibodies should be bound with the Fc portion to the polymeric surface). Large excess of ligand might be required or lack of sensitivity can be the consequence. As a remedy, surface modified beads have been developed, carrying chemical groups like carboxylic acid, amines, hydroxyl groups and others, which can be used for covalent coupling of biomolecules. Carboxylic groups on the bead surface for instance can be converted into a reactive NHS-ester, which readily reacts with amino groups from antibodies or amino modified oligonucleotides. Covalent coupling provides a more precise control of the coating

level, allows to work with lower coating concentrations of biomolecules, binds more protein compared to adsorption and the coated beads are thermally more stable.[74] Covalent coupling also allows the use of spacers between bead and attached biomolecule to improve sensitivity. An alternative to covalent coupling is the use of commercially available Nickel beads, which readily bind His-tagged biomolecules and biotinylated ligands can easily be bound to streptavidin-coated beads.

2.4.2 The Luminex Technology

The Luminex Lab Map Technology (Luminex, Austin, TX, USA) utilizes beads dyed with different amounts of fluorescent dye. Currently 100 different species of beads are available, which are impregnated with a distinct blend of two fluorescent dyes (Fig. 2.14). It is planned to use a third dye in the near future, resulting in 1,000 spectrally distinct species of beads. Luminex beads are available in a carboxylated format and with streptavidin coating. So-called universal array beads, which carry a unique zip-code for hybridization of nucleic acids have recently been introduced (FlexMAP Beads).

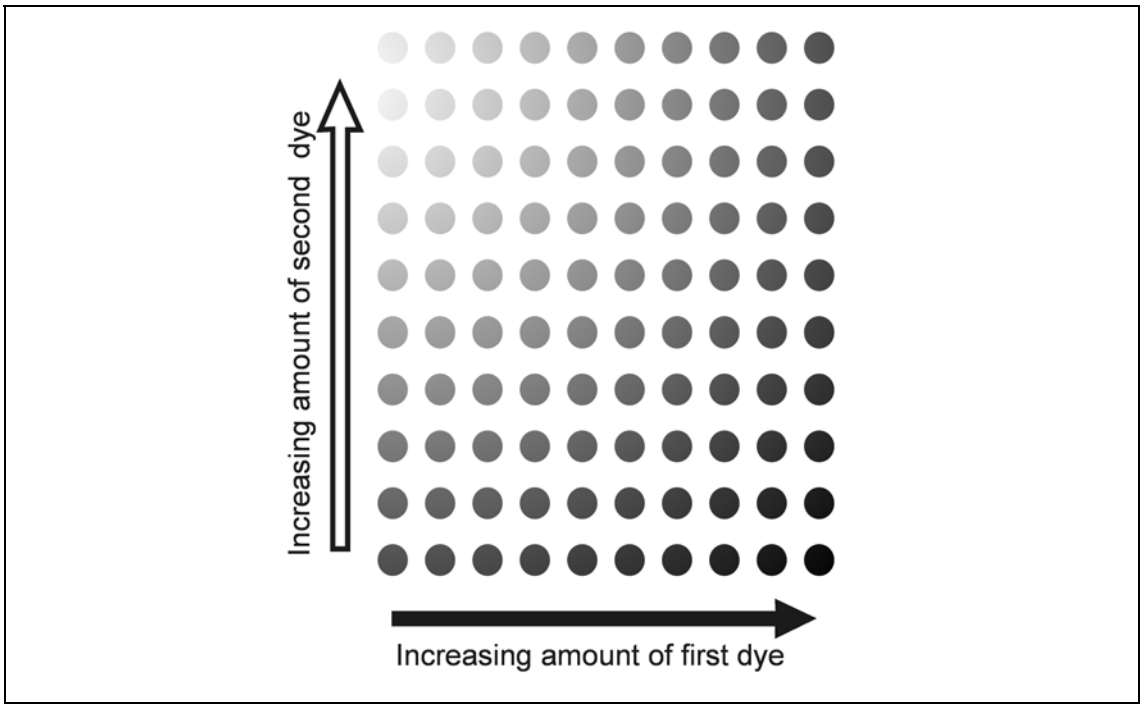

Fig. 2.14
Suspension array of 100 species of spectrally distinct color-coded microspheres. Beads are impregnated with a blend of two fluorescent dyes.

A conventional flow cytometer with three fluorescence channels, measures two dyes that specify the bead population and a reporter dye that specifies hybridization events. By using more sophisticated flow cytometers with additional fluorescent channels (e.g. FACSCalibur, BD Biosciences, San Diego, CA, USA), it would be possible to use more dyes for color-coding of beads or more colors for the test reaction. However, flow cytometers are expensive and their capabilities exceed what is required for bead-based

analysis. With the Luminex100 (Luminex, Austin, TX, USA) a low-cost flow cytometer (LX 100) is available that is specifically adapted to the measurement of color-coded beads and has only the size of a desktop PC. The instrument uses solid phase lasers and avalanche photo diodes replace photomultipliers for light scatter (635 nm) and two emission channels (645-669 nm and >712 nm). The excitation wavelength for the reporter dye has been increased to 532 nm to reduce Rayleigh stray light and to match the excitation of phycoerythrin, which is detected by a photomultiplier (Fig. 2.15) For the analysis of large numbers of samples, a Luminex HTS brings high-throughput capabilities.[75-77]

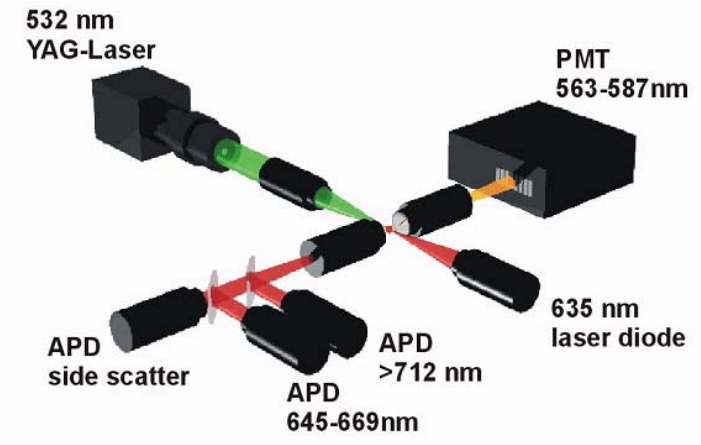

Fig. 2.15
Schematic of Luminex optics. The instrument uses solid phase lasers for excitation and avalanche photo diodes (APDs) replace photomultipliers for light scatter (635 nm) and two emission channels (645-669 nm and >712 nm). Reporter emission is detected at 532 nm by means of a photomultiplier (PMT).

2.4.3 Assay formats in nucleic acid testing

2.4.3.1 Direct hybridization

Several formats of wetware were adapted to bead-based nucleic acid testing. The simplest method is the direct hybridization of labeled nucleic acids to beads carrying a sequence specific oligonucleotide.

An assay for gene expression analysis using color-coded microspheres was developed using this technique. In this 'Beads Array for the Detection of Gene Expression' (BADGE), labeled cRNA was generated which was hybridized to a set of 20 bead populations carrying specific capture sequences of 25 base pairs for 20 *Arabidopsis* genes. The obtained expression profiles were similar to those obtained by Affymetrix GeneChip Analysis.[78]

In addition to expression profiling, sequence specific hybridization of labeled PCR product to microspheres has been performed. A sensitive multiplexed bead assay for

the detection of three viral nucleic acids (HIV, HSV and HCV) was developed. Here, labeled primers were used in amplification of the relevant viral nucleic acids, which were hybridized to sequence specific oligonucleotides bound to the beads.[79]

A third test applying the format of direct hybridization was a screening test for mutations in the CFTR gene, which can cause cystic fibrosis. A set of 10 bead population, each carrying a sequence specific oligonucleotide for 10 common mutations, and 10 bead populations, each carrying the corresponding wild-type sequence, were used. The relevant DNA segments were amplified by PCR using labeled primers prior to hybridization to the beads.[80]

2.4.3.2 Competitive hybridization

Since hybridization of longer PCR products to microspheres results in significant cross-talk and decreased sensitivity, a different assay format, the competitive hybridization, was applied. With this technique, allele specific, fluorescently labeled oligonucleotides (reporter oligonucleotides) are hybridized to the target amplicon. Afterwards, microspheres coated with oligonucleotides complementary to the reporter oligonucleotides are added and capture the remaining non-hybridized oligonucleotides. A decrease of fluorescence intensity compared to the negative control (inhibition of fluorescence) will report a positive test result. This assay format was used for example for a multiplexed hybridization assay to perform HLA-DQA1 tissue typing of PCR-amplified human genomic DNA.[81]

2.4.3.3 Zip code techniques

The simultaneous and efficient hybridization of oligonucleotides with different lengths and GC-contents is still a problem, even when using special hybridization buffers. In addition, the allelic discrimination of single nucleotide polymorphisms by hybridization is problematic due to the similarity of the sequences. To this end, the idea of using so-called zip codes was brought to light. Here, probes for allelic discrimination carry a 5'-dangling end with an artificial unique DNA sequence for hybridization to beads subsequent to allelic discrimination. This zip-codes are designed with identical length and GC content and limited cross-talk in simultaneous hybridization to their complementary sequence bound to the bead. An additional advantage is, that the development of novel assays does no longer require production of differently coated beads. With this assay format, three different techniques have been evaluated: the oligo ligation assay (OLA), minisequencing (also called: single base primer extension, SBCE) and the allele specific primer extension (ASPE).

In the OLA, two adjacent hybridized oligonucleotides are enzymatically ligated to each other using DNA ligase. This reaction only takes place, when the nucleotides next to the ligation position are fully complementary. The first (capture) oligonucleotide is

designed to hybridize to the target amplicon with its 3' base complementary to the polymorphic base. It also carries a 5'-dangling end with the zip code. The second (reporter) oligonucleotide hybridizes to the target sequence just adjacent to the 3' end of the capture probe and carries a fluorescent tag at its 3' end. If the capture probe matches exactly the SNP query position, the probes are ligated to each other, bringing a fluorescent tag to the capture oligonucleotide, which is detected by flow cytometry subsequent to hybridization to the bead populations. Two detection probes with different zip-codes are required to cover each of the two possible alleles. This method has been successfully applied in the analysis of 9 SNP markers located near the apo E locus on chromosome 19.[82]

In OLA, one additional, labeled reporter oligonucleotide is required for each variation. In minisequencing, only one primer for each allele is necessary, which is extended by one single labeled nucleotide terminator. The SBCE primer, carrying a zip-code at its 5'-dangling end, hybridizes to the target amplicon with its 3'-end one base upstream of the SNP query position. The primer is elongated by DNA polymerase using labeled dideoxynucleotide triphosphates (lacking a 3' hydroxyl group), so that only a single base – complementary to the polymorphic base being analyzed – is incorporated. The disadvantage of the SBCE format is, that up to four different label are required in multiplexing assays to cover each ddNTP and hence a sophisticated flow cytometer with six fluorescence channels would be necessary. The alternative is to use a separate reaction for each type of terminator nucleotide. Then, the analysis can be performed on a standard flow cytometer, but up to four different reactions are required in a multiplex assay to cover all possible nucleotides. This method has been successfully performed with 55 randomly selected SNPs near the apo E locus.[83]

In allele specific primer extension only one reaction is required for genotyping. Here, like in OLA, one oligonucleotide is designed for each allele to hybridize to the target amplicon with its 3' base complementary to the polymorphic base and a 5'-dangling end with a unique zip code. Standard deoxynucleotides are used, with one type of them (e.g. dCTP) being labeled. A DNA polymerase lacking 3'-5' exonuclease activity is used to elongate primers with a perfect match at their 3' end. During elongation, dependent on the sequence several labels are incorporated which increases sensitivity compared to formats incorporating only one label (like OLA or SBCE). After the genotyping reaction, the zip-coded primers are hybridized to color-coded beads (Fig. 2.16). Only one fluorescent channel is required for detection of each possible genotype and no labeled oligonucleotides are required, which are expensive. This format seems to be the most suited and cost-efficient method for bead-based nucleic acid testing of SNPs and mutations. For evaluation, 20 random selected SNPs have successfully been analyzed in 633 probands applying this method.[84]

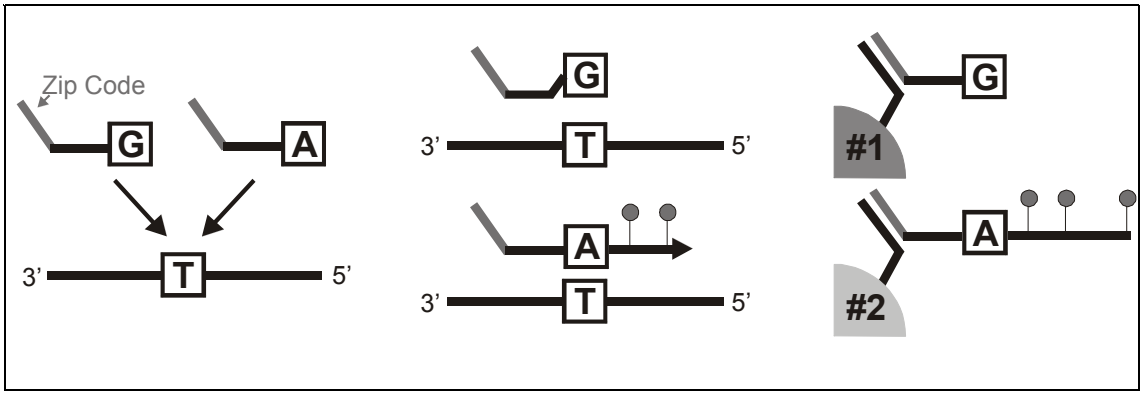

Fig. 2.16
Principle of the ASPE reaction. One oligonucleotide is designed for each allele to hybridize to the target amplicon carrying a 5'-dangling end with a unique zip code. Only primers with a perfect match at their 3' end are elongated by DNA polymerase incorporating several labeled dNTPs. After the genotyping reaction, the zip-coded primers are hybridized to color-coded beads.

A set of 100 color-coded beads carrying unique zip codes (FlexMAP Beads) is available as platform for the development of bead-based nucleic acid tests (Luminex, Austin, Texas). Several commercially available kits (Tm Bioscience, Toronto, Canada) have been developed with this platform, applying the ASPE format, including multiplex tests for screening of sequence variations in genes relevant for blood coagulation (Factor V, Prothrombin, MTHFR), drug metabolism (CYP450-2D6) and cystic fibrosis (CFTR).[85, 86]

2.4.3.4 Benefits of bead-based technology

At this time, bead-based nucleic acid testing might not be able to compete with high-density DNA arrays in gene expression profiling and with the determination of more precise expression levels using homogeneous real time PCR methods. However, it has been shown, that bead-based assays produce accurate results in low density gene expression profiling and sequence detection. In terms of the analysis of sequence variations, especially genotyping of single nucleotide polymorphisms and point-mutations, bead-based technology provides a real alternative to conventional methods due to its flexibility and high multiplexing capability. The individual combination and the simultaneous determination of complete parameter profiles might be more cost-efficient than conventional single analysis methods and so could find their way into laboratory diagnostics. Flow cytometry is a robust and automatable platform for bead-based testing. A large set of commercially available zip code beads (Luminex FlexMAP beads) provides an ideal condition for the development of new bead-based nucleic acid tests. With allele-specific primer extension, an optimal wetware has been established, allowing high specificity, increased sensitivity and thus requiring a less sophisticated platform at the same time.

3 Materials and methods

3.1 Patients

Patient A (individual II-1 from pedigree A, see Fig. 3.1) is a 40-year old woman with HDL levels of 125-145 mg/dl with fluctuations of 20-30 mg/dl resulting from the use of contraceptives and during menstrual cycle. She had spontaneous abortions due to trophoblast dysplasia and a family history of thrombosis and congestive heart failure. Lipid parameters prior to lipid lowering therapy are shown in Fig. 3.1. As can be seen in pedigree A, her sister (II-2) and her mother (I-2) had also elevated cholesterol but HDL was normal.

One 50-year old female (patient B) had very low HDL (2 mg/dl) and apo A-I (<4 mg/dl) levels. She suffered from polyneuropathy, xanthelasmas in both eyes and gingiva hyperplasia. She underwent tonsillectomy, but showed no signs of coronary artery disease or splenomegaly.

Another patient (C) with very low HDL (4 mg/dl) and apo A-I levels was a 61-year old male with abdominal aorta aneurysm and arcus lipoides who had suffered myocardial infarction at the age of 57.

Patient D (individual II-2 of pedigree D) is a third patient with analphalipoproteinemia (HDL 2 mg/dl, apo A-I 5 mg/dl) originating from England. As can bee seen from pedigree D (Fig. 3.1), his sister and his father also had reduced HDL and apo A-I levels, but the lipid status of the mother seemed normal.

Pedigree E (Fig. 3.1) consists of four members originating from the Middle East (Iran) with a low-HDL-syndrome associated with cardiac hypertrophy and myopathy and a co-segregation of glucose-6-P-dehydrogenase (G6PD) deficiency. At the time of diagnosis, the father, a 60-year old man presented with normal plasma lipids under lipid-lowering treatment and a disturbed glucose tolerance. Xanthelasms had been known since the age of 24. There is a family history of myocardial infarction and vascular disease. Hypertrophic cardiomyopathy has been diagnosed about 6 years ago and arrythmias are existent since the age of 30 years. Dystonia and myopathy of the right arm and hand were also reported since puberty. The 53-year old mother (I-2) had reduced lipid parameters. She is homozygous for the Mediterranean (Ser188Phe) variant of Favism (G6PD deficiency). The index patient (patient E, individual II-1), her 19-year old daughter (II-2) had significant myopathy of the eyelids (ptosis), clinical signs of favism and low HDL-levels (30 mg/dl). Her 30-year old daughter (II-1) had low HDL levels (27 mg/dl) as well. The Mediterranean (Ser188Phe) mutation was detected in the heterozygous form in both siblings, whereas the father has a wild-type G6PD genotype.

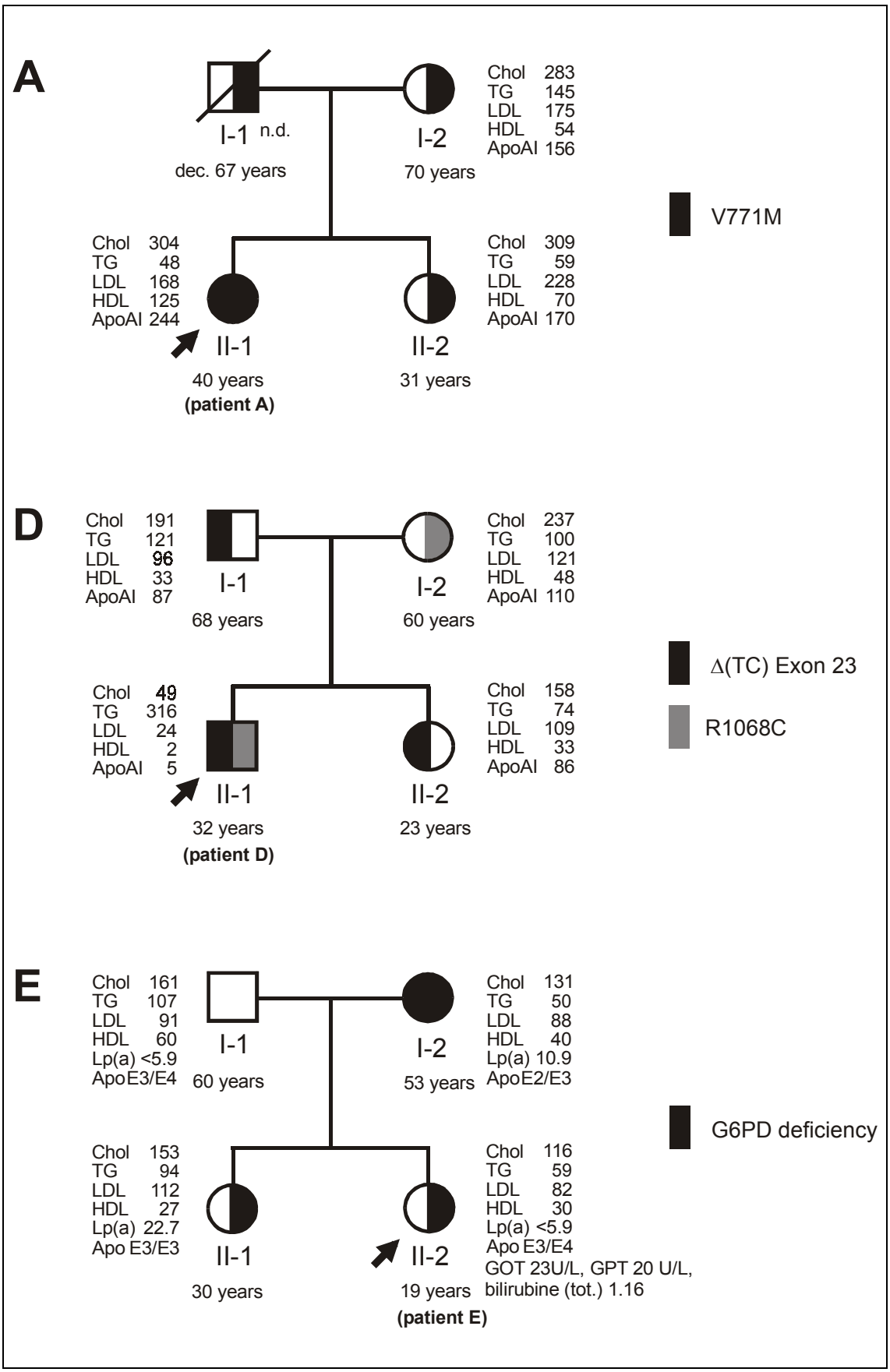

Fig. 3.1
Pedigree A shows a patient with extremely high HDL (cholesterol levels prior to statin therapy). Pedigree D illustrates a family with a known Tangier mutation Δ(TC). Pedigree E shows a family with low cholesterol levels and co-segregation of Mediterranean form of G6PD deficiency.

3.2 DNA isolation from EDTA blood

Genomic DNA was extracted from whole blood EDTA samples using QIAamp Blood DNA Midi Kit (Qiagen, Hilden, Germany) or MagNa Pure LC DNA Isolation Kit using the MagNa Pure LC extraction robot (Roche Molecular Biochemicals, Mannheim, Germany). Extraction yielded up to 260 µl of DNA with a maximum concentration of 180 ng/µl. For single samples, the quality of the DNA was checked by agarose gel and the concentration was determined by absorption measurement. DNA was normalized to a concentration of 10 ng/µl. For large numbers of DNA samples for archivation, a BIOMEK FX pipeting robot was used (Beckman Coulter, Fullerton, CA, USA). The DNA was pipeted into microtiter plates, the concentration was determined using PicoGreen Assay (Molecular Probes, Eugene, OR, USA) and the DNA was normalized to a concentration of 10 ng/µl. Normalization was carried out using BFX-Normalization Software and Biomek FX pipeting robot (Beckman Coulter, Fullerton, CA, USA).

3.3 Study groups for polymorphism identification

The German cohorts consisted of 476 healthy blood-donors from the University Hospital Regensburg (DON) as control group, as well as 614 patients with coronary artery disease (CAD). In addition, 508 German octogenarians (OCT) partly from the Berlin Aging Study (436 individuals) and partly recruited by our laboratory (72 individuals) were available. From a large cohort of patients with type 2 diabetes mellitus (TTDM), collected in 30 dialysis centers in South Germany, 384 individuals were randomly selected.

Also, a group of individuals with high HDL (High-HDL) and a group with low HDL (Low-HDL) values were constituted. Individuals of these groups were patients of the University Hospital, as well as selected participants of the RESPECT study (Risk Evaluation and Stroke Prevention in the Elderly), which were individuals between 65 and 80 years with high or low HDL levels prior to statin therapy. Additional samples with high HDL were collected in Austria and some individuals with low HDL were from the Netherlands. Of all available probands, the samples with the highest and lowest HDL values were selected. In this study, high HDL was defined as above 100 mg/dl and low HDL as below 50 mg/dl.

The Hungarian cohorts consisted of 105 healthy blood donors (DON_H), 153 patients with coronary artery disease (CAD_H) and 246 patients who had suffered from stroke (STROKE_H).

The Italian cohorts were constituted from 191 healthy blood donors (DON_I) as the reference group and 130 centenarians of age 100 and older (CENT).

3.4 Sequence of ABCA1

The sequences used for analysis and numbering of ABCA1 sequence variations were obtained from GeneBank (www.ncbi.nlm.nih.gov). The genomic DNA sequence was derived from accession number AF275948, mRNA and codon assignment was according to NM005502 (mRNA). ABCA1 promoter sequence and numberings relative to transcription start point is derived from Langmann et al.[34]

3.5 DNA sequencing of PCR products from ABCA1

DNA sequencing was performed with an ABI Prism 3100 Genetic Analyzer with Big Dye Terminator technology (ABI, Foster City, CA, USA). PCR products from ABCA1 were generated with the set of primers listed in Tab. 7.4 of the appendix (see page 89), which were purchased from MWG (Ebersberg, Germany) in HPSF grade purity. For the screening approach, the promoter region including exon 1 was sequenced in a larger set of individuals using promoter primer ABCA1-pro2-f and the reverse primer from exon 1 (ABCA1-ex1-r). Here, a longer capillary was used in the sequencer for improved read-out of increased fragment lengths.

In selected individuals, the complete ABCA1 gene (except introns) was analyzed. Here, the promoter was sequenced using primer pairs ABCA1-pro1-f/r, ABCA1-pro2-f/r and ABCA1-pro3-f/r. The sequence of all 50 exons was analyzed using primer pairs ABCA1-ex1-f/r to ABCA1-ex50-f/r.

CoreKit (Qiagen, Hilden, Germany) was used for all PCR reactions. The reaction mixture contained 1x PCR-Buffer, 1x Q-Solution, 200 µM each dNTP, 0.4 µM each Primer, 0.05 U/µl Taq polymerase and approximately 0.5 ng/µl genomic DNA. The reaction mixture with a final volume of usually 50 µl was processed in a PE 9600 GeneAmp PCR System thermal cycler (ABI, Foster City, CA, USA) with 5 min of initial denaturation, followed by 40 cycles of 45 sec at 95°C, 30 sec at 60°C and 30 sec at 60°C. Final extension was performed for 10 min at 72°C. After PCR, the obtained product was purified using QIAquick spin columns (Qiagen, Hilden, Germany) and agarose gel electrophoresis was performed.

The cycle sequencing reaction was performed using BigDye Terminator Kit 1.1 (ABI, Foster City, CA, USA). The final volume of 20 µl contained 4 µl of BigDye Terminator Mix RR-2500, 4 µl of 5x sequencing buffer, 3.2 pmol (3.2 µl of 1 pmol/µl) of either forward or reverse primer, and 1 to 8.8 µl of purified PCR-product, depending on concentration of PCR product as roughly determined by agarose gel electrophoresis. The final product was purified using Centrisep spin columns (ABI, Foster City, CA, USA), vacuum-dried, dissolved in 20 µl of Hi-Di formamide (ABI, Foster City, CA, USA), and processed in the ABI 3100 Genetic Analyzer.

Sequence analysis was done using Sequencher software (GeneCodes, Ann Arbor, MI, USA) by comparing the patient derived sequences to a reference sequence from the database. Trimmed sequences were aligned to each other or to the wild-type sequence. All electronically reported sequence variations and heterozygosities were checked individually in the original electropherogram.

3.6 Fluorescent fragment analysis of VNTR polymorphisms

PCR was carried out with appropriate primer pairs (ABCA1-pro3-f/r and ABCA1-pro4-f/r), of which one primer was labeled with 6-FAM at its 5'-end. The same concentrations and conditions for PCR as presented in 3.4 were used. The PCR product was diluted with water (1:7), then 3 µl of it were pooled with 1 µl of ROX-labeled size standard Genscan-500 (ABI, Foster City, CA, USA) and supplemented with 20 µl of Hi-Di formamide. The mixture was analyzed on an ABI Prism 3100 Genetic Analyzer with Gene Scan software.

3.7 Electrophoretic mobility shift assay (gel shift assay)

HeLa cells were harvested by centrifugation, washed once with ice-cold PBS, and washed twice with wash buffer composed of 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). After resuspension in 1 ml ice-cold wash buffer, cells were centrifuged 1 min at full speed in a microfuge. Hypotonic buffer containing 0.1% Nonidet P-40 was added to lyse the cell pellet. After 5 min incubation on ice, nuclei were pelleted in a microfuge for 15 min at 4°C. Nuclei were resuspended in lysis buffer containing 20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 10% v/v glycerol, and incubated at 4°C for 15 min with gentle vortexing. Subsequently, the nuclear debris was pelleted by centrifugation at 4°C for 15 min and the supernatant was diluted 1:6 with storage buffer composed of 20 mM HEPES, pH 7.9, 0.05 M KCl, 0.2 mM EDTA, 0.5 mMDTT, 0.5 mM PMSF, and 20% v/v glycerol. The extracts were aliquoted and stored at -70°C. [87]

An equivalent of 40,000 cpm of double-stranded [^{32}P] end-labeled oligonucleotide probe containing either double stranded oligonucleotide -210F-wt (5'-TCC ACC CCC ACC CCA CCC CAC CCA CCT CCC CCC-3') or -210R-mut (5'-TCC ACC CCC ACC CCA CCC ACC TCC CCC C-3') was incubated with 10 µg of nuclear extract from the HeLa cells in a buffer containing 50 mM HEPES/HCl, pH 7.9, 6 mM $MgCl_2$, 50 mM dithiothreitol, 100 µg/ml bovine serum albumin, 0.01% Nonidet P-40, and 2 µg of poly(dI-dC) (Amersham Biosciences, Freiburg, Germany) at room temperature for 20 min. In competition experiments, nuclear extracts were pre-incubated with a 100-fold molar excess of competitor for 10 min prior to the addition of the radiolabeled

probe. DNA-protein complexes were resolved on a native 8% polyacrylamide gel and audioradiographed with Kodak BioMax MR films at 80°C.

3.8 Reporter gene assay with ABCA1 promoter

Genomic DNA served as a template for the amplification of a 600 (595) bp-fragment of the promoter sequence including the VNTR-ZNF-polymorphism. Forward primer -381F with *Nhe*I restriction site (5'-GGG GCT AGC CAA ATT CCA CTG GTG CCC TTG-3') and reverse primer +214R with *Bgl*II restriction site (5'-GGG AGA TCT TTT CCA CCT TTG TGT TTG CGT CTC) were used in PCR with Taq PCR Core Kit (Qiagen, Hilden, Germany) and standard protocol. The reporter constructs from two individuals each homozygous for one form of the VNTR-polymorphism (595 bp, 600 bp), as verified by fragment analysis, were cloned by ligation of PCR fragments into the *Bgl*II and *Nhe*I restriction sites of the pGL3-basic vector. A promoterless pGL3-basic vector served as negative control, while the pGL3-control vector, which contains the SV40 early promoter, was used as positive control. RAW264.7 cells were transiently transfected with Fugene reagent (Roche Molecular Biochemicals, Mannheim Germany) as described by the manufacturer. Two µg of the respective reporter gene constructs were co-transfected with 1 µg of the pSV β-galactosidase vector in order to normalize differences in transfection efficiency. Cells were harvested 24 hours after transfection and lysed in reporter lysis buffer (Promega, Madison, WI, USA). Luciferase assay reagent containing Luciferyl-CoA was added after centrifugation. Luciferase activity was determined in a LUMAT LB9501 (Berthold). The β-galactosidase enzyme assay (Promega) was used for the determination of β-galactosidase activity. Each experiment was repeated three times with two distinct plasmid preparations and measurements were done in triplicate. The experiment was repeated with co-transfection of up to 4 µg of ZNF202m1 vector containing nucleotide positions 8-1960 (GeneBank accession number AF027219) cloned into pcDNA3.1/V5/His-Topo plasmid (Invitrogen Carlsbad, CA, USA).[87]

3.9 LightCycler SNP analysis

Hybridization probes were purchased from TIB MOLBIOL (Berlin, Germany), which were labeled with fluorescent dyes FITC and LC Red 640 or LC Red 705, respectively. Primers with HPSF quality were obtained from MWG (Ebersberg, Germany). The standard reaction mixture (20 µl) contained 1x LightCycler DNA Master Hybridization Probes (Roche Molecular Biochemicals, Mannheim, Germany), 4 mM $MgCl_2$, 0.5 µM each primer and 0.2 µM each probe when using LC Red 640 or 0.4 µM when using LC Red 705, respectively. In the amplification step, for each assay, 40 cycles of 94°C (0 sec), 57°C (10 sec) and 72°C (15 sec), were performed.

3.10 TaqMan SNP analysis

Assays based on the hydrolysis probes from TaqMan were usually run as 5 µl reactions on a ABI Prism 7900 HT instrument in 384-well plates. The probes specific for the individual SNP were labeled either with VIC- or FAM-fluorophore and with a non-fluorogenic quencher (NFQ) and a minor groove binder (MGB) for better allelic discrimination. PCRs were cycled in 384-well microtiter plates in the TaqMan (ABI Prism 7900 HT) or externally in conventional block thermocyclers MWG Primus (MWG, Ebersberg, Germany). Endpoint fluorescent data acquisition was achieved using the TaqMan instrument. All assays were adapted to the standard protocol: 0.2 µM each probe and 0.9 µM each primer in 1x Universal PCR Master Mix. All reagents were from ABI (Foster City, CA, USA). The program for thermal cycling was identical for all assays (40 cycles of 15 sec at 95°C and 60 sec at 60°C in the amplification step).

3.11 Microspheres

FlexMAP microspheres were purchased from Luminex Corp. (Austin, TX, USA). These carboxyl modified beads were 5.5 µm in diameter and were color-coded with two fluorescent dyes. Beads were coated with unique isothermal “anti-tag” sequences (zip codes). The “tag” sequences (complementary “anti-tag” sequences) are presented in Tab. 3.1. Oligonucleotides that should be hybridized to FlexMAP beads must carry these taq sequences at their 5’ end. The microspheres were stored in TRIS-EDTA buffer (10 mM TRIS, 1 mM EDTA) at a concentration of 2.5×10^5 microspheres per ml. Bead-based assays were analyzed on Luminex LX100 analyzer with XY platform and IS software.

3.12 Statistical data analysis

Pearson’s χ^2 test was performed to check for differences in distribution of wild-type, homozygous and heterozygous individuals in cohorts compared to the control group (healthy blood donors). Differences were considered significant, when $p(\chi^2)$ was 0.05 or below.

Observed allelic frequencies were also checked for Hardy-Weinberg equilibrium by Fisher’s exact test. A frequency distribution is considered to be significantly different from Hardy-Weinberg, when p_{HWE} is 0.05 or below.

For matched pair data, McNemar test was performed instead of normal χ^2 test.

To indicate the level of statistical significance, values are marked with a single asterisk (*) for $p \leq 0.05$, a double asterisk (**) for $p \leq 0.01$, and a triple asterisk (***) for $p \leq 0.001$.

No	Zip Code Sequence	No	Zip Code Sequence
01	CTT TAA TCT CAA TCA ATA CAA ATC	51	TCA TTT CAA TCA ATC ATC AAC AAT
02	CTT TAT CAA TAC ATA CTA CAA TCA	52	TCA ATC ATC TTT ATA CTT CAC AAT
03	TAC ACT TTA TCA AAT CTT ACA ATC	53	TAA TTA TAC ATC TCA TCT TCT ACA
04	TAC ATT ACC AAT AAT CTT CAA ATC	54	CTT TTT CAA TCA CTT TCA ATT CAT
05	CAA TTC AAA TCA CAA TAA TCA ATC	55	TAT ATA CAC TTC TCA ATA ACT AAC
06	TCA ACA ATC TTT TAC AAT CAA ATC	56	CAA TTT ACT CAT ATA CAT CAC TTT
07	CAA TTC ATT TAC CAA TTT ACC AAT	57	CAA TAT CAT CAT CTT TAT CAT TAC
08	AAT CCT TTT ACA TTC ATT ACT TAC	58	CTA CTA ATT CAT TAA CAT TAC TAC
09	TAA TCT TCT ATA TCA ACA TCT TAC	59	TCA TCA ATC AAT CTT TTT CAC TTT
10	ATC ATA CAT ACA TAC AAA TCT ACA	60	AAT CTA CAA ATC CAA TAA TCT CAT
11	TAC AAA TCA TCA ATC ACT TTA ATC	61	AAT CTT ACC AAT TCA TAA TCT TCA
12	TAC ACT TTC TTT CTT TCT TTC TTT	62	TCA ATC ATA ATC TCA TAA TCC AAT
13	CAA TAA ACT ATA CTT CTT CAC TAA	63	CTA CTT CAT ATA CTT TAT ACT ACA
14	CTA CTA TAC ATC TTA CTA TAC TTT	64	CTA CAT ATT CAA ATT ACT ACT TAC
15	ATA CTT CAT TCA TTC ATC AAT TCA	65	CTT TTC ATC AAT AAT CTT ACC TTT
16	AAT CAA TCT TCA TTC AAA TCA TCA	66	TAA CAT TAC AAC TAT ACT ATC TAC
17	CTT TAA TCC TTT ATC ACT TTA TCA	67	TCA TTT ACT CAA CAA TTA CAA ATC
18	TCA AAA TCT CAA ATA CTC AAA TCA	68	TCA TAA TCT CAA CAA TCT TTC TTT
19	TCA ATC AAT TAC TTA CTC AAA TAC	69	CTA TAA ACA TAT TAC ATT CAC ATC
20	CTT TTA CAA TAC TTC AAT ACA ATC	70	ATA CCA ATA ATC CAA TTC ATA TCA
21	AAT CCT TTC TTT AAT CTC AAA TCA	71	ATC ATT ACA ATC CAA TCA ATT CAT
22	AAT CCT TTT TAC TCA ATT CAA TCA	72	TCA TTT ACC TTT AAT CCA ATA ATC
23	TTC AAT CAT TCA AAT CTC AAC TTT	73	ATC AAA TCT CAT CAA TTC AAC AAT
24	TCA ATT ACC TTT TCA ATA CAA TAC	74	TAC ACA TCT TAC AAA CTA ATT TCA
25	CTT TTC AAT TAC TTC AAA TCT TCA	75	AAT CAT ACC TTT CAA TCT TTT ACA
26	TTA CTC AAA ATC TAC ACT TTT TCA	76	AAT CTA ACA AAC TCA TCT AAA TAC
27	CTT TTC AAA TCA TAT CTC AAC TTT	77	CAA TTA ACT ACA TAC AAT ACA TAC
28	CTA CAA ACA AAC AAA CAT TAT CAA	78	CTA TCT ATC TAA CTA TCT ATA TCA
29	AAT CTT ACT ACA AAT CCT TTC TTT	79	TTC ATA ACT ACA ATA CAT CAT CAT
30	TTA CCT TTA TAC CTT TCT TTT TAC	80	CTA ACT AAC AAT AAT CTA ACT AAC
31	TTC ACT TTT CAA TCA ACT TTA ATC	81	CTT TAA TCT ACA CTT TCT AAC AAT
32	ATT ATT CAC TTC AAA CTA ATC TAC	82	TAC ATA CAC TAA TAA CAT ACT CAT
33	TCA ATT ACT TCA CTT TAA TCC TTT	83	ATA CAA TCT AAC TTC ACT ATT ACA
34	TCA TTC ATA TAC ATA CCA ATT CAT	84	TCA ACT AAC TAA TCA TCT ATC AAT
35	CAA TTT CAT CAT TCA TTC ATT TCA	85	ATA CTA CAT CAT AAT CAA ACA TCA
36	CAA TTC ATT TCA TTC ACA ATC AAT	86	CTA ATT ACT AAC ATC ACT AAC AAT
37	CTT TTC ATC TTT TCA TCT TTC AAT	87	AAA CTA ACA TCA ATA CTT ACA TCA
38	TCA ATC ATT ACA CTT TTC AAC AAT	88	TTA CTT CAC TTT CTA TTT ACA ATC
39	TAC ACA ATC TTT TCA TTA CAT CAT	89	TAT ACT ATC AAC TCA ACA ACA TAT
40	CTT TCT ACA TTA TTC ACA ACA TTA	90	CTA AAT ACT TCA CAA TTC ATC TAA
41	TTA CTA CAC AAT ATA CTC ATC AAT	91	TTC ATA ACA TCA ATC ATA ACT TAC
42	CTA TCT TCA TAT TTC ACT ATA AAC	92	CTA TTA CAC TTT AAA CAT CAA TAC
43	CTT TCA ATT ACA ATA CTC ATT ACA	93	CTT TCT ATT CAT CTA AAT ACA AAC
44	TCA TTT ACC AAT CTT TCT TTA TAC	94	CTT TCT ATC TTT CTA CTC AAT AAT
45	TCA TTT CAC AAT TCA ATT ACT CAA	95	TAC ACT TTA AAC TTA CTA CAC TAA
46	TAC ATC AAC AAT TCA TTC AAT ACA	96	ATA CTA ACT CAA CTA ACT TTA AAC
47	CTT CTC ATT AAC TTA CTT CAT AAT	97	AAT CTC ATA ATC TAC ATA CAC TAT
48	AAA CAA ACT TCA CAT CTC AAT AAT	98	AAT CAT ACT CAA CTA ATC ATT CAA
49	TCA TCA ATC TTT CAA TTT ACT TAC	99	AAT CTA CAC TAA CAA TTT CAT AAC
50	CAA TAT ACC AAT ATC ATC ATT TAC	100	CTA TCT TTA AAC TAC AAA TCT AAC

Tab. 3.1
Zip code sequences to be appended to ASPE primer at 5' end. The complementary sequence ("Anti-Tag" sequence) is attached to the correponding region of FlexMAP Bead.[88]

4 Objective

SNPs build the essence of our genetic diversity. Therefore, the identification of relevant markers and the efficient analysis of these small sequence variations in our genome are of major importance for effective medical treatment and preventive medicine.
ABCA1 has recently been identified as the main regulator for plasma HDL cholesterol, which has a protective effect in the development of arteriosclerosis and related diseases (such as coronary heart disease), sequence variations in this gene could be responsible for altered levels of HDL cholesterol and hence predisposition to the development of these diseases.
The aim of this work is to screen for novel sequence variations of relevance in the ABCA1 gene and to develop assays for variations of potential interest for diagnostic and epidemiological studies. Existing formats and instrumentation for their use in SNP genotyping will be compared and evaluated. A high-throughput laboratory for the efficient analysis of huge numbers of samples should be set up and tested for genotyping of several large cohorts using the newly developed assays. Finally, a multiplex assay system for a novel bead-based platform for the simultaneous determination of genotypes will be set up and evaluated.

5 Results

5.1 Screening for functional sequence variations and mutations in ABCA1

In order to identify new sequence variations of potential relevance, a knowledge-based approach was pursued by analyzing (1) the promoter region and exon 1, (2) selective regions of ABCA1 (exons 49 and 50) in a large number of individuals and (3) the complete ABCA1 gene in a few individuals with abnormal HDL-levels for the following reasons:
(1) Sequence variations in promoters are good candidates to be responsible for altered regulation of gene expression. The prevalence of such polymorphisms may be different in various study populations. The first exon of ABCA1 is non-coding and its sequence may also contribute to the regulation of ABCA1 gene expression. In order to find sequence variations, this region was sequenced of 248 individuals from four study groups: 63 healthy octogenarians, 62 healthy blood donors, 62 individuals with low HDL and 62 individuals with high HDL. Healthy blood donors are regarded as a representative cross-section of the population, so this cohort should display the normal

distribution of genotypes in the population. However, these mostly quite young people still could develop diseases and aberrant lipid levels if they harbor genotypes that predispose such a development. In contrast, very old people with healthy phenotype should have “good genes” and sequence variations should accumulate which have protective effects to develop diseases such as cardiovascular disease as well as normal or good HDL levels. If sequence variations encountered during screening are related to HDL levels, a different distribution of genotypes should be found in the cohorts with low and high HDL. Since ABCA1 is involved in cholesterol efflux, changes in ABCA1-expression should have impact on perturbations in lipoprotein profiles and affect disease predisposition. The promoter region of ABCA1 is under investigation and contains binding sites for several transcription factors, one of them being ZNF202. Sequence variations near or at such a binding site might well influence the expression of ABCA1 and be of diagnostic or therapeutical value.

(2) It has been shown that the C-terminal amino acids of ABCA1, which are part of the cytoplasmatic terminal tail, interact with various proteins and thus participate and manipulate intracellular processes resulting in aberrant phenotypes [45]. Therefore exons 49 and 50 of the ABCA1 gene have been analyzed, which code for 127 amino acids (amino acids 2,135-2,261). In order to find relevant polymorphisms in those region, both exons in the individuals from the four study groups described above.

(3) Because patients with severely diminished HDL-levels usually have mutations in the ABCA1 gene, it seems conceivable that individuals with extremely aberrant HDL-levels (very high or very low) will display sequence variations in ABCA1 that are not present or are less frequent in healthy controls. So far, 21 different homozygous or compound-heterozygous mutations in ABCA1 have been reported in 31 patients with analpha-lipoproteinemia (Tangier Disease). By identifying new mutations and correlating the location of the mutations with the patients‘ epicrisis, it might be possible to elucidate important domains in the transporter.

5.1.1 Sequencing of ABCA1 promoter region and exon 1

In order to identify novel polymorphisms that may have effects on the expression of ABCA1, sequencing of ABCA1 promoter and (non-coding) exon 1 of many individuals has been performed. In this screening, along with 3 known SNPs (C1176G, G1355C and C1487G [89, 90]), 3 novel SNPs compared to database entry AF275948 have been identified (G1047C, C1152T and C1440T). In addition, a VNTR polymorphism, designated VNTR-ZNF, was found in the screening approach (see below). Frequencies of all encountered SNPs are presented in Tab. 5.1 and reflect the frequencies as have been identified in the sequenced individuals, which are homozygous for one form of VNTR-ZNF. Due to scrambling of the DNA sequence in a few VNTR-heterozygous individuals, proper genotyping of these samples was not possible without cloning of

PCR products. Therefore, the presented frequencies may not reflect frequencies in the general population and Hardy-Weinberg equilibrium cannot be applied.

The statistical data analysis (Tab. 5.1) revealed, that the G1047C (G-395C) exchange was significantly more frequent in the Low-HDL cohort (18% homozygotes and 55% heterozygotes) compared to the High-HDL cohort (27% homozygotes and 24% heterozygotes). The exchange C1152T (C-290T) was found at a significantly higher prevalence in Low-HDL (5% homozygotes, 25% heterozygotes) than in DON (5% homozygotes, 10% heterozygotes) or octogenarians (10% homozygotes, 5% heterozygotes). The C1440T (C-7T) exchange was found significantly increased in Low-HDL (9% homozygotes, 45% heterozygotes) compared to DON (10% homozygotes, 18% heterozygotes) and octogenarians (14% homozygotes and 10% heterozygotes). The known polymorphisms C1176G and G1355C were found signifycantly decreased in High-HDL compared to DON and Low-HDL.

Two additional polymorphisms in simple repeat motifs were found (VNTR polymorphisms). In the repeat motif GTTTTT GTTT GTTT GTTTT GTTT GTTT (Pos. 674-700), along with the wild-type sequence, two variations Δ(GTTT) and Δ(GTTTTGTTT) were identified. This polymorphism was designated “VNTR-SRY”, since it is located at a putative SRY binding site. Since the SRY gene is located on the Y-chromosome, this polymorphism was investigated further in male probands of DON, High- and Low-HDL cohorts. Another variation, Δ(ACCCC), was found in the simple repeat motif ACCCCC ACCCC ACCCC ACCC ACC (Pos. 1,216-1,238), which is located at a putative ZNF202 binding site, therefore the polymorphism was designated “VNTR-ZNF”. Since ZNF202 is known to down-regulate ABCA1-expression, further functional investigation of the VNTR-ZNF was performed. All encountered sequence variations are illustrated in Fig. 5.1.

Further functional testing of the VNTR-ZNF polymorphism was performed. Allele frequency data in various study groups should help to elucidate a possible role in specific phenotypes. A 380-bp fragment of the promoter containing the VNTR-ZNF polymorphism using forward primer CCC TAA GAC ACC TGC TGT ACC C (ABCA1-pro3-f) with 5’-FAM modification and reverse primer CTG AGA ACC GGC TCT GTT GGT (ABCA1-pro3-r) was generated. Separation of PCR products was done by capillary electrophoresis (fragment analysis) on 119 individuals: 53 healthy blood donors, 39 probands with hyperalpha- and 27 probands with hypoalphalipoproteinemia. Results are presented in Tab. 5.2 and show an increased prevalence of the wild-type sequence in patients with high HDL-C-levels. The non-deleted form of the VNTR is more frequent in the individuals with high-HDL (70.4%) compared to the reference group (DON) and the low-HDL group (54.4% and 57.2%, respectively), however the difference is not significant.

		DON	OCT	Low-HDL	High-HDL	Total
number of individuals		62	56	63	51	232
Promoter						
G1047C*	het	45%	27%	55%	24%	38%
(G-395C)	hom	21%	25%	18%	27%	23%
				p=0.003**		
C1152T*	het	10%	5%	25%	14%	13%
(C-290T)	hom	5%	10%	5%	4%	6%
			p=0.001***			
		p=0.010**				
C1176G[89]	het	26%	17%	41%	6%	23%
(C-266G)	hom	21%	25%	18%	27%	23%
				p=0.000***		
		p=0.019*				
VNTR-ZNF Δ1222-6* (Ins-220)	hom	5%	8%	7%	4%	6%
G1355C[90]	het	22%	31%	35%	6%	24%
(G-92C)	hom	17%	9%	7%	8%	10%
				p=0.002**		
			p=0.001***			
		p=0.001***				
C1440T*	het	18%	10%	45%	25%	23%
(C-7T)	hom	10%	14%	9%	16%	12%
			p=0.000***			
		p=0.001***				
Exon 1						
C1487G[90]	het	1%	6%	11%	12%	7%
(C+41G)	hom	3%	1%	0%	0%	1%

Tab. 5.1
Prevalences (in %) of heterozygous and homozygous polymorphisms in ABCA1 promoter and exon 1. Novel sequence variations are marked with an asterisk. The remaining percentage represents wild-type individuals. p-values are given for significant differences in distribution of genotypes only. Numberings are based on accession number AF275948, numberings in parentheses refer to the position relative to transcription start point.[34] OCT, octogenarians; DON, healthy blood donors.

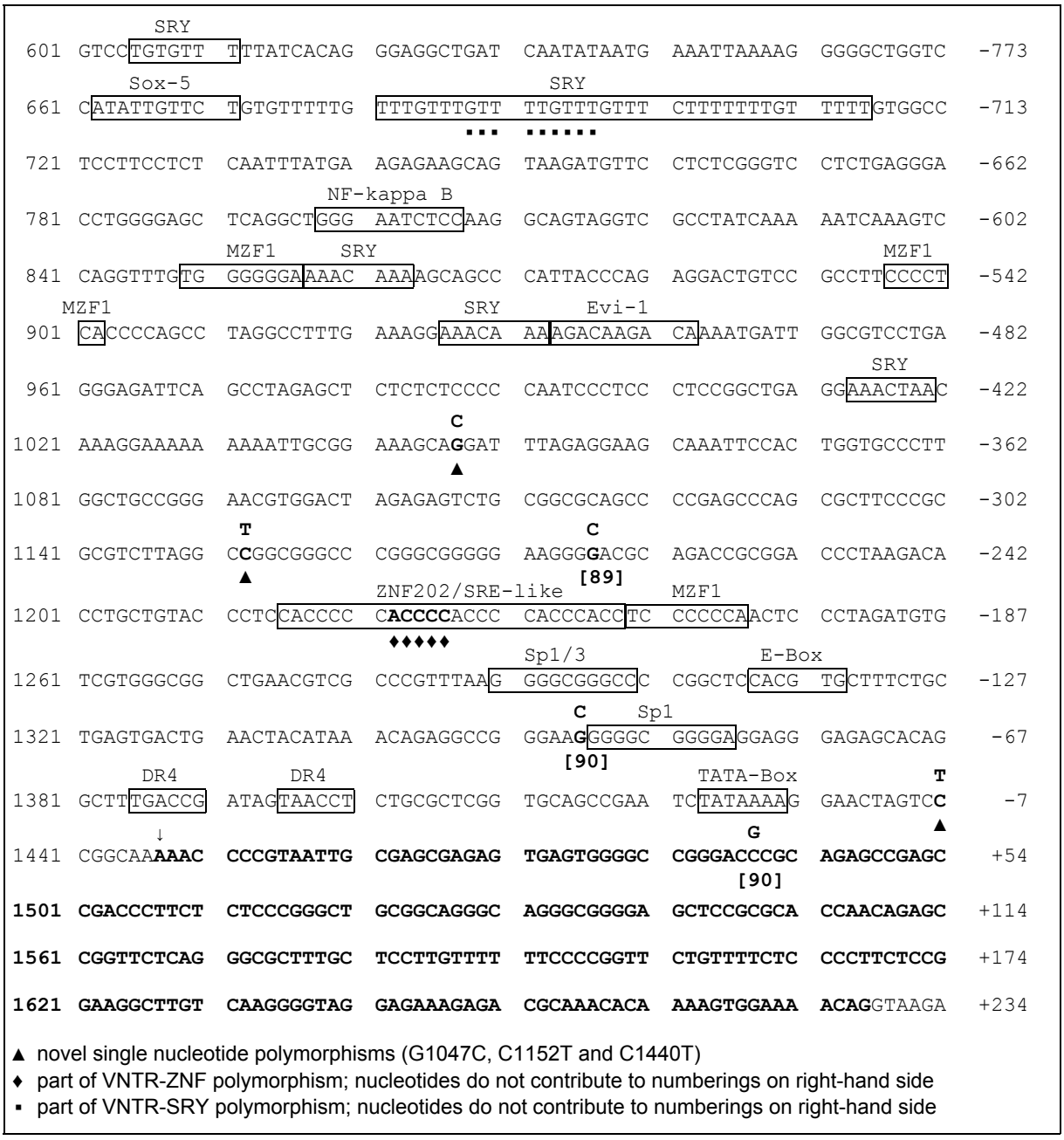

Fig. 5.1
ABCA1 promoter sequence and polymorphisms therein. Numberings on the left refer to accession number AF278948, numberings on the right reflect the distances relative to the transcription start point according to Langmann et al.[34].

	DON	High-HDL	Low-HDL	Total
Individuals	46	27	21	94
HDL-C	66±14	121±14	40±9	76±33
wt	54.3%	70.4%	57.2%	59.6%
het	41.3%	18.6%	33.3%	33.0%
Δ1,222-6	4.4%	11.1%	9.5%	7.4%
$p(\chi^2)$	–	0.103	0.637	–

Tab. 5.2
Frequencies of VNTR-ZNF in various study groups and mean HDL levels. p-values are calculated compared to DON. An increased prevalence of wild-type homozygotes was found in patients with high HDL-C-levels, however the differences are not significant.

An in vitro gel shift assay with two labeled double stranded oligonucleotides, derived from the “wild-type” and “mutant” (shorter) form of the VNTR, was performed. As can be seen in Fig. 5.2, the shorter form of the VNTR polymorphisms (Δ1,222-1,226) was bound more efficiently by transcription factors of the nuclear extracts from HeLa cells. Lane 1 and 2 show the free probe of mutant (1) and wild-type (2) oligonucleotide. Lane 3 and 4 contain mutant (3) and wild-type (4) oligonucleotide incubated with 5 µg of nuclear extract. Competition with 100-fold excess of unlabeled wild-type oligonucleotide reversed the binding while reversal was not as efficient with the mutant oligonucleotide (data not shown).

In a reporter gene assay, performed in RAW264.7 cells, using the two forms of the VNTR polymorphism as part of the ABCA1 promoter and luciferase gene, a significant difference with and without co-transfection with ZNF202 vector could not be detect (data not shown).

Frequencies of the VNTR-SRY polymorphism were determined by fragment analysis in males only, since the SRY gene is located on the Y-chromosome. A 246 bp fragment of the promoter region containing the VNTR polymorphism at a SRY binding site (VNTR-SRY) was amplified using forward primer 5’-GGA GGT CTG GAG TGG CTA CAT-3’ (ABCA1-pro4-f) and labeled reverse primer 5’-FAM-AGA GGA CCC GAG AGG AAC AT (ABCA1-pro4-r). No significant differences in prevalence were found in the three study groups. Therefore the VNTR-SRY polymorphism was not characterized further. The data are presented in Tab. 5.3.

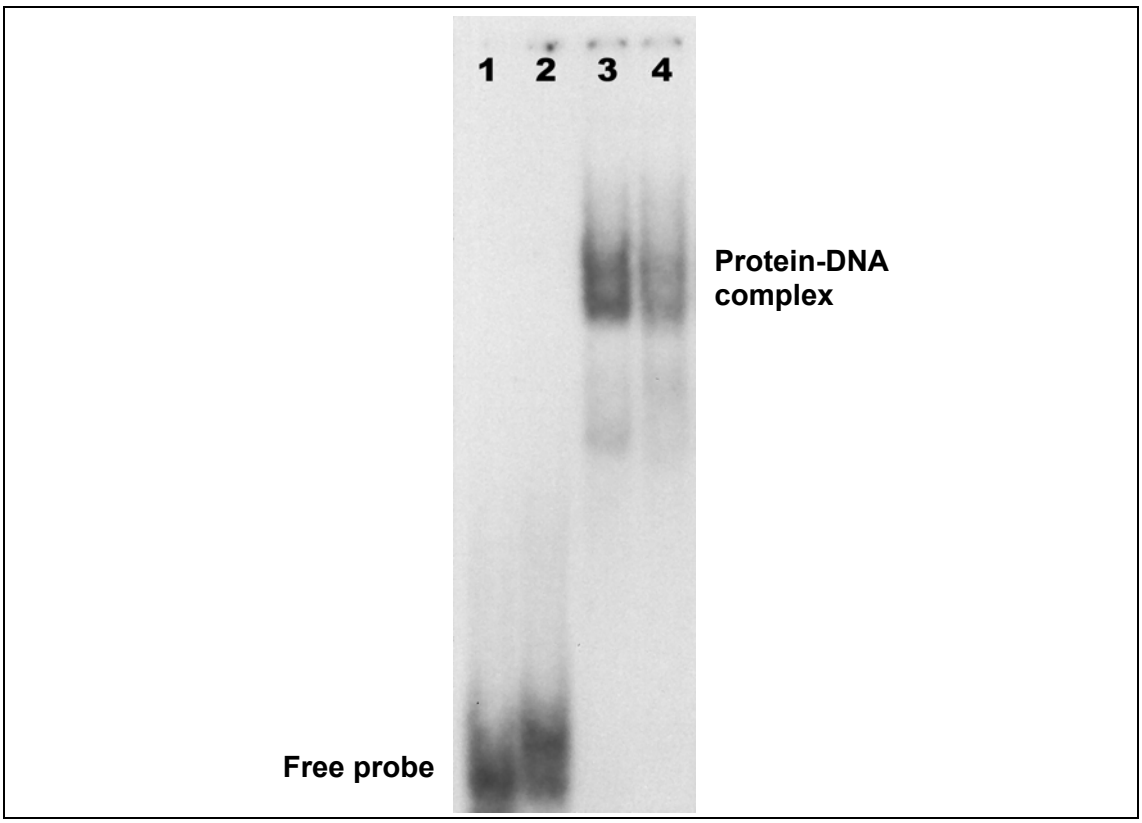

Fig. 5.2
Electromobility shift assay. Lane 1, free probe of mutant (Δ1222-1226) oligonucleotide; lane 2, free probe of wild-type oligonucleotide; lane 3, mutant oligonucleotide incubated with 5 µg nuclear extract; lane 4, wild-type oligonucleotide incubated with 5 µg nuclear extract.

	DON	High-HDL	Low-HDL	Total
Individuals	50	33	44	221
HDL-C	57±12	93±16	35±7	59±25
Homozygotes:				
wt	2.0%	12.1%	6.8%	6.3%
Δ(GTTT)	8.0%	3.0%	2.3%	4.7%
Δ(GTTTTGTTT)	18.0%	21.2%	29.5%	22.8%
Heterozygotes:				
wt / Δ(GTTT)	12.0%	15.2%	11.4%	12.6%
wt / Δ(GTTTTGTTT)	24.0%	15.2%	15.9%	18.9%
Δ(GTTT) / Δ(GTTTTGTTT)	34.0%	33.3%	34.1%	33.9%
Allele frequencies:				
wt	20.0%	27.3%	20.5%	22.0%
Δ(GTTT)	32.0%	27.3%	25.0%	28.3%
Δ(GTTTTGTTT)	47.0%	45.5%	54.5%	49.2%
p(χ^2)	–	0.541	0.516	–

Tab. 5.3
Frequencies of VNTR-SRY in various study groups and mean HDL levels (males only). p-values are calculated compared to DON. No significant differences were detected.

5.1.2 Sequencing of exons 49 and 50 of the ABCA1 gene

Since the C-terminal amino acids of ABCA1, which are part of the cytoplasmatic terminal tail, interact with various proteins and thus participate and manipulate intracellular processes [45], sequence variations in this region could be of major importance. Therefore, exons 49 and 50 of the ABCA1 gene have been sequenced, which code for 127 amino acids (amino acids 2,135-2,261) in the individuals from the four study groups described above. Sequencing of 63 healthy octogenarians, 62 healthy blood donors, 62 individuals with low HDL and 62 individuals with high HDL revealed only one sequence variation, resulting in amino acid exchange P2150L (C143444T) which has been reported earlier by Clee et al.[15] One individual (healthy octogenarian) was found to be heterozygous for this sequence variation.

5.1.3 Sequencing of the complete ABCA1 gene in individuals with aberrant HDL levels

Individuals with extremely aberrant HDL-levels (very high or very low) are expected to display sequence variations in ABCA1 that are not present or are less frequent in healthy controls. Therefore, the complete coding region and the promoter of several individuals has been sequenced.

In patient A (individual II-1 in pedigree A, Fig. 3.1) a G102555A (V771M) polymorphism in a homozygous state was identified. No further sequence variations of potential interest were found. Sequencing of additional genes possibly leading to high HDL did not reveal any mutation that might account for the high HDL in this individual (CETP, SR-BI, LDL receptor). The V771M polymorphism was analyzed in the DON cohort. It was found that 451 (94.4%) out of 478 successfully genotyped individuals were wild-types, only 27 (5.6%) were heterozygous V/M771 and no homozygous M771 individual was found.

Sequencing of the complete ABCA1 gene of the 50-year old female with 2 mg/dl HDL (patient B) showed that she was heterozygous for A107009G (N935S), a known Tangier mutation [17], and also heterozygous for the novel mutation T103822A, which results in the amino acid exchange W840R. The DON cohort was genotyped for this variation and no R840 variation was found in 570 successfully determined individuals.

In the 61-year old male (patient C) the novel mutation G98481T was identified in a heterozygous state, which results in amino acid exchange W590L. A mutation in the same codon (W590S) has already been described in a previous analyzed Tangier patient in our laboratory.[17] The patient was also heterozygous for two known polymorphisms (R219K and I883M), but no second mutation could be identified so far. The L590 allele was not detected in 570 individuals of the DON cohort.

Sequencing of the complete ABCA1 gene in the proband of pedigree D (Fig. 3.1) revealed, that the patient is heterozygous for a two base-pair deletion (ΔTC) in exon 23

affecting position 1 and 2 in codon 1,114, leading to a premature stop codon, following out-of-frame translation, in codon 1145. This mutation was found in the original kindred with HDL deficiency from Tangier Island.[91] In addition, patient D was heterozygous for a novel sequence variation in exon 22, which results in the amino acid exchange arginine to cysteine at codon 1,068 (R1068C). Three other known sequence variations have been found (heterozygous R219K, heterozygous E1172D and homozygous R1587K). Sequencing of exons 22 and 23 in the other family members revealed that the father and the sister were heterozygous for the Tangier mutation. This concurs with the reduced HDL and apo A-I levels in those individuals. The mother was found heterozygous for amino acid exchange R1068C (see pedigree D, Fig. 3.1).

Because of the low-HDL-syndrome, which was obvious in both daughters of pedigree E (Fig. 3.1), the complete ABCA1 gene was sequenced in individual II-2 (patient E). Several sequence variations were identified, with two of them located within exon 49 (F2163S) and exon 50 (V2244I) near the COOH terminus of ABCA1. Analysis of the rest of the family focusing on the identified polymorphisms revealed that the mother is heterozygous for F2163S and the father is heterozygous for V2244I. Both variations are present in heterozygous form also in her sister and may account for their low HDL-cholesterol. This is supported by the fact that one of the enzymes that was identified in the yeast two hybrid system to interact with the COOH-terminus of ABCA1 is part of the succinate dehydrogenase complex (iron sulfur complex), which is known to be involved in the pathogenesis of hypertrophic cardiomyopathy [45]. The S2163 allele was not found in 544 successfully genotyped individuals of the DON group, the I2244 variation could not be detected in 473 successfully determined donors.

Tab. 5.4 summarizes the sequence variations or mutations that have been identified within the coding region of the ABCA1 gene leading to amino acid changes and their origin within the individuals analyzed. Fig. 5.3 shows the location of these variations in the ABCA1 protein product. For observed single nucleotide variations in the coding region, affecting amino acid sequence, assays based on hydrolysis probe technology (TaqMan) were established to clarify if these variations are polymorphisms or point mutations. With one exception (V771M), none of these SNPs was found in more than 500 individuals of the DON cohort. Therefore, it can be concluded, that V771M represents a SNP, while all other variations are (point-) mutations. The primers and probes that have been developed for TaqMan analysis are presented in Tab. 5.5, which can be used with the same conditions specified in 3.10. The development of TaqMan assays and their processing in high-throughput workflow will be described in more detail in the next section.

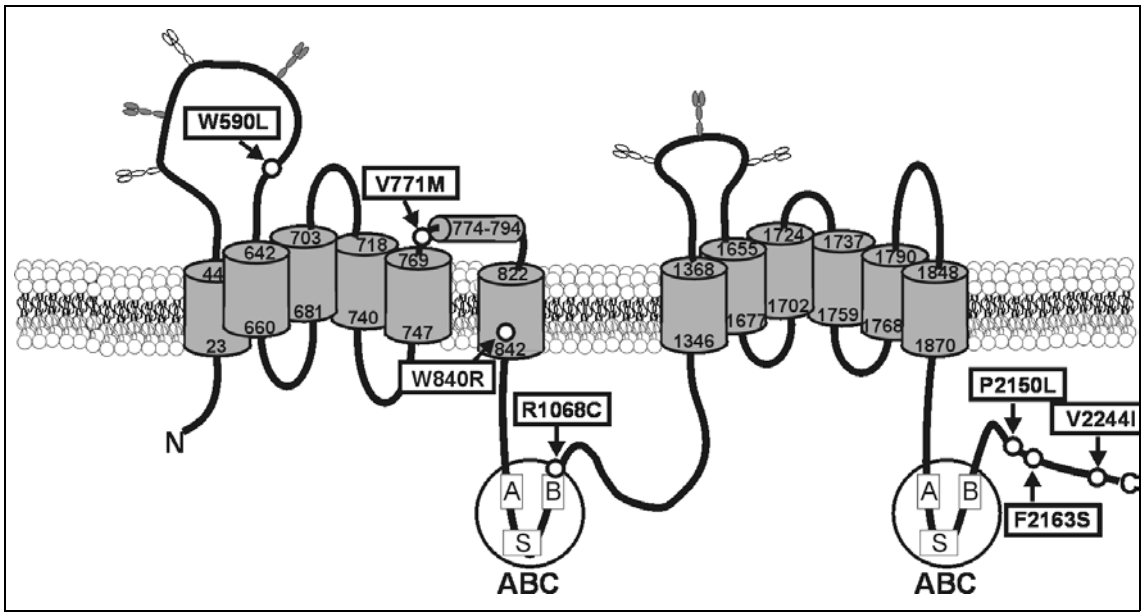

Fig. 5.3
Location of sequence variations in the coding region of ABCA1. The amino acid position and structure is according to Tanaka et al.[92] Black dots indicate mutations in the Tangier patients or relevant sequence variations.

Exon	Amino acid	Nucleotide	Pos. DNA AF275948	Pos. mRNA NM005502	phenotype of patient
14	W590L	T**G**G→T**C**G	98,481	2,082	HDL deficiency (C)
16	V771M	**G**TG→**A**TG	102,555	2,624	increased HDL (A)
17	W840R	**T**GG→**A**GG	103,822	2,831	HDL deficiency (B)
22	R1068C	**C**GC→**T**GC	109,904	3,515	HDL deficiency (D)
49	F2163S	T**T**T→T**C**T	143,483	6,801	low HDL and G6PD deficiency (E)
50	V2244I	**G**TT→**A**TT	144,665	7,043	

Tab. 5.4 Sequence variations found in ABCA1 and phenotypes of patients.

W590L (G2082C)	TM-W590L-f	5‘-AGC TGA CCC CTT TGA GGA CAT-3‘
	TM-W590L-r	5‘-CTC CAC CAC ATC CTG CAA GTA G-3‘
	TM-W590-vic	5‘-**VIC**-CCC CC**C** AGA CGT A-**MGB**-**NFQ**-3‘
	TM-L590-fam	5‘-**FAM**-CCC CC**G** AGA CGT A-**MGB**-**NFQ**-3‘
V771M (G2624A)	TM-V771M-f	5‘-GGC ATC ATC TAC TTC ACG CTG TA-3‘
	TM-V771M-r	5‘-CAG AGG TAC TCA CAG CGA AGA TCT T-3‘
	TM-V771-vic	5‘-**FAM**-TGT GAA GCC CA**C** GTA G-**MGB**-**NFQ**-3‘
	TM-M771-fam	5‘-**VIC**-TGA AGC CCA **T**GT AGT C-**MGB**-**NFQ**-3‘
W840R (T2831A)	TM-W840R-f	5‘-GCT GTT TGA CAC CTT CCT CTA TGG-3‘
	TM-W840R-r	5‘-TGT ACC TGG AAA GAC AGC CTC AA-3‘
	TM-W840-vic	5‘-**VIC**-TGT ACC **A**GG TCA TCA C-**MGB**-**NFQ**-3‘
	TM-R840-fam	5‘-**FAM**-TGT ACC **T**GG TCA TCA C-**MGB**-**NFQ**-3‘
P2150L (C6762T)	TM-P2150L-f	5‘-TTC AGG TTT GGA GAT GGT TAT ACA ATA G-3‘
	TM-P2150L-r	5‘-GAA ATG CAA GTC CAA AGA AAT CCT-3‘
	TM-P2150-vic	5‘-**VIC**-CAA CC**C** GGA CCT GA-**MGB**-**NFQ**-3‘
	TM-L2150-fam	5‘-**FAM**-CAA CC**T** GGA CCT GAA-**MGB**-**NFQ**-3‘
F2163S (T6801C)	TM-F2163S-f	5‘-AAG CCT GTC CAG GAT TTC TTT G-3‘
	TM-F2163S-r	5‘-CAT GTT CCG GTG TTT CTC TTT TAG-3‘
	TM-F2163-vic	5‘-**VIC**-CCA GGA **A**AT GCA AGT C-**MGB**-**NFQ**-3‘
	TM-S2163-fam	5‘-**FAM**-CAG GA**G** ATG CAA GTC-**MGB**-**NFQ**-3‘
V2244I (G7043A)	TM-V2244I-f	5‘-ATG ATG ACC ACT TAA AAG ACC TCT CA-3‘
	TM-V2244I-r	5‘-GCT TTC TTT CAC TTT CTC ATC CTG TAG-3‘
	TM-V2244-vic	5‘-**VIC**-TGG AC**G** TTG CAG TTC-**MGB**-**NFQ**-3‘
	TM-I2244-fam	5‘-**FAM**-AGT AGT GGA C**A**T TGC-**MGB**-**NFQ**-3‘

Tab. 5.5
Primers and probes for TaqMan analysis of novel polymorphisms in the coding region of ABCA1.

5.2 High-throughput genotyping of ABCA1 polymorphisms

In this part, a concept for high-throughput analysis of single nucleotide polymorphisms has been established using TaqMan technology integrated in a roboting platform. Since the previously analyzed promoter region contains numerous polymorphisms, partly in close proximity to each other, this region was not chosen for the development of assays, to avoid difficult primer design and laborious troubleshooting of assays. The main focus of this part was the establishment of the high-throughput technology. Therefore, four interesting polymorphisms in the coding region of the ABCA1 gene have been selected, which affect amino acid sequence: R219K, I883M, V771M and E1172D (the location of these polymorphisms is presented in Fig. 5.4). The R219K polymorphism has been associated with reduced severity of arteriosclerosis, fewer coronary events, decreased triglycerides and a trend to increased HDL in men with coronary heart disease.[93, 94] The M771 variant is suspected to be involved in the cause of increased HDL levels in one subject (see 5.1.3), but no association has been

shown so far. The M883 allele is associated with significantly higher plasma HDL cholesterol in Canadian Inuit and Japanese.[95, 96] The D1172-allele has been found significantly increased in CHD end points.[97] Several cohorts were subject to genotyping with the new established workflow. Before the high-throughput concept could be achieved, genotyping was carried out using LightCycler melting curve analysis. Data obtained from this method will also be included in the association study, since both methods are reliable and firmly established. In addition, since experience has been gained with both methods, LightCycler and TaqMan, a direct comparison and evaluation can be performed.

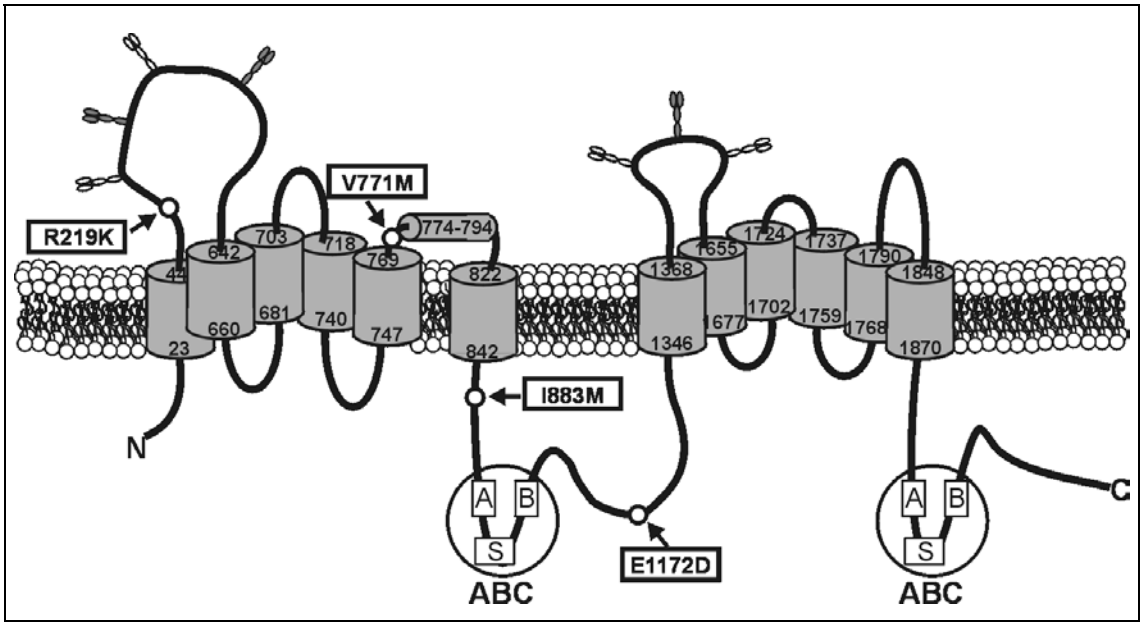

Fig. 5.4
Structure of the ABCA1 protein product and location of four selected polymorphisms therein.

The selected cohorts constituted for analysis were healthy people older than 80 years (OCT), patients with premature onset of coronary artery disease (CAD), stroke (STROKE) and others. The distribution of genotypes in these groups can be compared to a cohort of healthy blood donors (DON). If the polymorphisms investigated, have an effect on HDL levels or other related lipid parameters, an unequal distribution of genotypes should be observed in the different cohorts. E.g., if one allele had a positive effect on lipid parameters or a protective effect in aging, it should be more abundant in very old people. Since HDL has a protective effect on the development of arteriosclerosis and HDL levels are influenced by a certain polymorphism, a different distribution of genotypes could be observed in cohorts with related diseases, such as the cohort of patients with coronary artery disease or stroke. A group of patients with diabetes mellitus type 2 (TTDM) was also analyzed, since low HDL cholesterol and

apolipoprotein AI levels are often found in association with other cardiovascular risk factors, including the type 2 diabetes mellitus.[98]

In addition a large set of samples from Hungary (_H) and Italy (_I) were available. It might also be possible to find an unequal distribution of genotypes in populations with different ethnical origin.

5.2.1 LightCycler SNP analysis

Primers and hybridization probes for SNPs in ABCA1 for LightCycler melting curve analysis were designed manually or using primer3 software [99]. With 3 LightCycler instruments, running up to 10 times per day for genotyping of large cohorts, 960 samples could be processed per day. Genotypes were obtained applying the melting curve analysis inherent of the LightCycler technology. Genotyping had to be done manually by checking each melting curve plot manually and recording the data into Excel spreadsheets. The designed primers and probes were used with standard LightCycler protocols (see 3.9). Primers and probes are given in Tab. 5.6, typical metling curve plots for each assay are presented in Fig. 5.5.

R219K (G969A)	LC-R219K-f	5‘-TTT TGC AAG GCT ACC AGT TAC A-3‘
	LC-R219K-r	5‘-CAG GAT TGG CTT CAG GAT GT-3‘
	LC-R219-det	5‘-**LCRed705**-GCC TAC CAA **G**GG AGA AAC TG-(P)-3‘
	LC-R219-anc	5‘-CTT GGT GAC CAA GAA GTT TCT GAG CTT TG-**FITC**-3‘
V771M (G2624A)	LC-V771M-f	5'- TAC AAG TGA GTG CTT GGG ATT -3'
	LC-V771M-r	5'- CCC ATT GGA AAA GAC AAT CAT C -3'
	LC-V771-det	5‘-**LCRed640**-GCA GGA CTA C**G**T GGG CTT C-(P)-3‘
	LC-V771-anc	5‘-CTG-TAC CTG CCC TAC TGC CTG TGT GTG GCA-**FITC**-3‘
I883M (A2962G)	LC-I883M-f	5‘-AGG CCC TGG TAT TTT CCT TG-3‘
	LC-I883M-r	5‘-CCC TGG AGT GGT TTC ACA GT-3‘
	LC-I883-det	5‘-**LCRed640**-CTT TCT GA**T** ATT CTC TTC TGG-(P)-3‘
	LC-I883-anc	5‘-TTA AAG AAA GAG CAG GAG GTC AAC AGC ACT-**FITC**-3‘
E1172D (G3829C)	LC-E1172D-f	GGA CAG TGT TTC TCA GAG CAG
	LC-E1172D-r	5‘AGC AGC AAA CCT TGA GTC AG
	LC-E1172-det	5‘-**LCRed640**-CGT GTC ACT **C**TC ATG GTC G-(P)-3‘
	LC-E1172-anc	5‘-AGA AAC CCC AGA GTC CTT ACC GAT GGT C-**FITC**-3‘

Tab. 5.6
Primers and hybridization probes for LightCycler

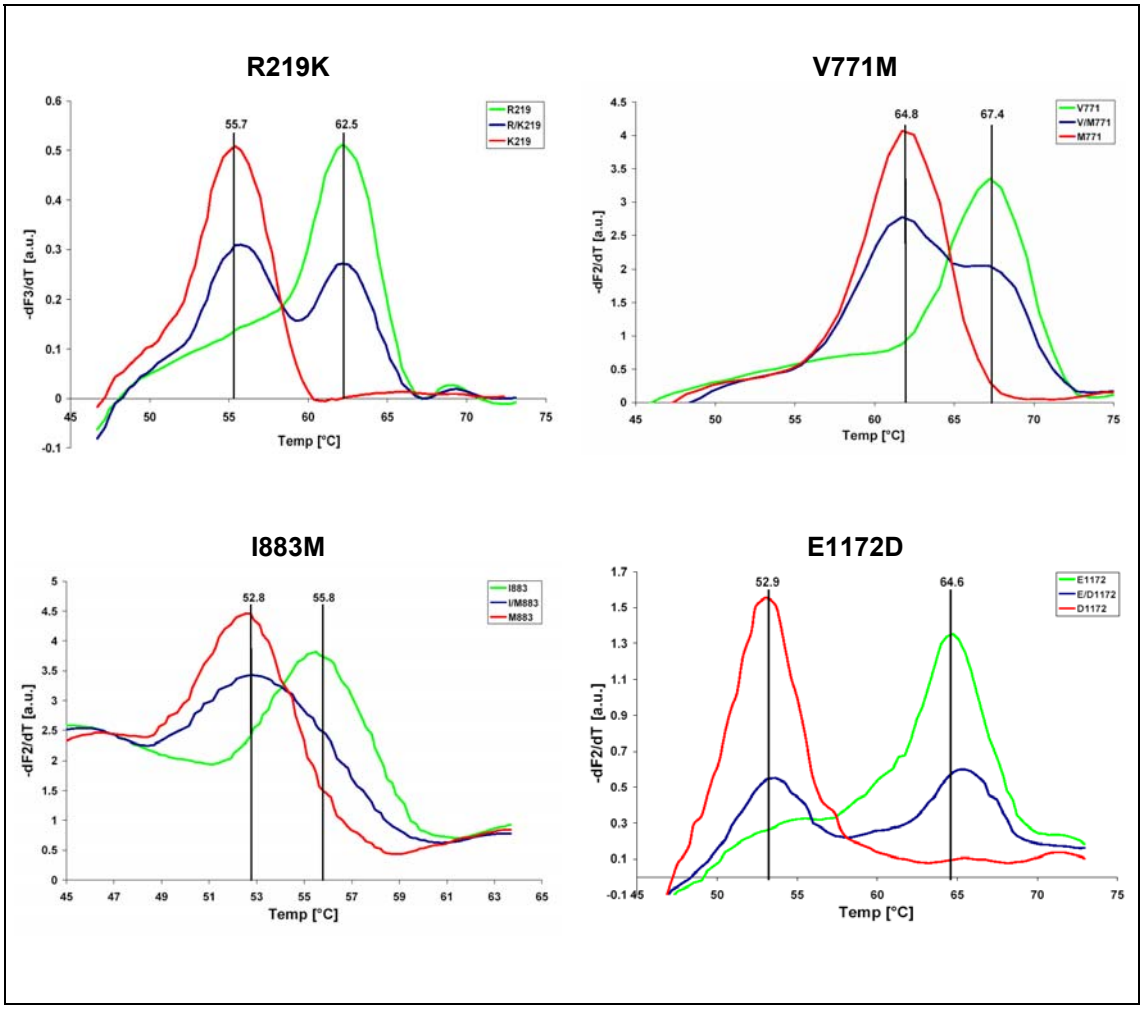

Fig. 5.5
Typical melting curve analysis plots for LightCycler assays R219K, V771M, I883M and E1172D.

5.2.2 TaqMan SNP analysis

TaqMan assays were run as 5 µl reactions on an ABI Prism 7900 HT instrument in 384-well plates. The probes specific for the individual sequence were labeled either with VIC or FAM-fluorophore at their 5'-end and with a non-fluorogenic quencher (NFQ) and a minor groove binder (MGB) for better allelic discrimination at the 3'-end. In the high-throughput phase the PCRs were cycled externally in 384-well microtiter plates on up to six conventional block thermocyclers (MWG, Ebersberg, Germany) and endpoint fluorescent data acquisition was achieved on the ABI Prism 7900 HT. Results were gated manually by the operator. The TaqMan was integrated in the high-throughput workflow as described later. Primers and hydrolysis probes were designed manually, using primer3 software [99], Primer Express (ABI, Foster City, CA, USA) or ABI's Assay-by-Design service. Primers and probes were used with the standard ABI protocol for TaqMan SNP analysis (see 3.10). Typical discrimination plots for the developed assays are presented in Fig. 5.6, primers and probes are listed in Tab. 5.7.

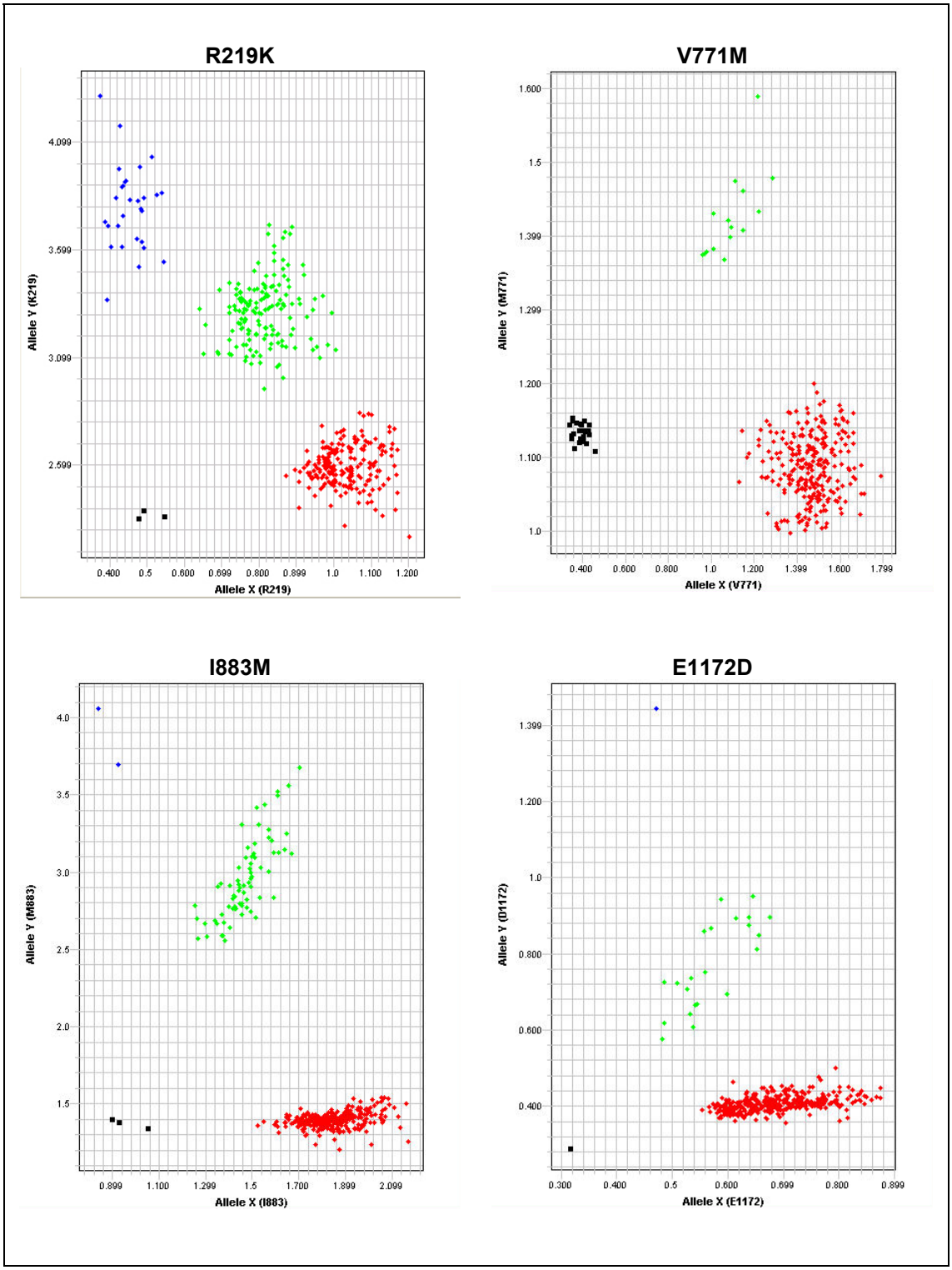

Fig. 5.6
Typical discrimination plots for TaqMan assays R219K, V771M, I883M and E1172D.

R219K (G969A)	TM-R219K-f	5‘-TGA CCA AGA AGT TTC TGA GCT TTG T-3‘
	TM-R219K-r	5‘-TGT CCA TGT TGG AAC GAA GTA CTC-3‘
	TM-R219-vic	5‘-**VIC**-ACC AA**G** GGA GAA AC-**MGB-NFQ**-3‘
	TM-K219-fam	5‘-**FAM**-ACC AA**A** GGA GAA ACT-**MGB-NFQ**-3‘
V771M (G2624A)	TM-V771M-f	5‘- GGC ATC ATC TAC TTC ACG CTG TA-3‘
	TM-V771M-r	5‘- CAG AGG TAC TCA CAG CGA AGA TCT T-3‘
	TM-V771-vic	5‘-**FAM**-TGT GAA GCC CA**C** GTA G-**MGB-NFQ**-3‘
	TM-M771-fam	5‘-**VIC**-TGA AGC CCA **T**GT AGT C-**MGB-NFQ**-3‘
I883M (A2962G)	TM-I883M-f	5‘-AGA GCC ACC CTG GTT CCA A-3‘
	TM-I883M-r	5‘-AAA GAA AGA GCA GGA GGT CAA CAG-3‘
	TM-I883-vic	5‘-**VIC**-CTT ACT TTC TGA **T**AT TCT-**MGB-NFQ**-3‘
	TM-M883-fam	5‘-**FAM**-CTT TCT GA**C** ATT CTC-**MGB-NFQ**-3‘
E1172D (G3829C)	TM-E1172D-f	5‘-GGA CAG TGT TTC TCA GAG CAG TTC T-3‘
	TM-E1172D-r	5‘-CCC AGA GTC CTT ACC GAT GGT-3‘
	TM-E1172-vic	5‘-**VIC**-CGA CCA TGA **G**AG TGA-**MGB-NFQ**-3‘
	TM-E1172-fam	5‘-**FAM**-CCA TGA **C**AG TGA CAC G-**MGB-NFQ**-3‘

Tab. 5.7
Primers and hydrolysis probes for TaqMan.

5.2.3 High-throughput workflow

For the fast analysis of a large number of samples the main tasks outlined in Tab. 5.8 have to be performed in an automated workflow. Subsequent to DNA isolation of a multitude of EDTA blood samples, DNA concentrations have to be determined and DNAs need to be normalized to a standardized concentration for archivation in appropriate large volume storage plates. After archivation of all DNAs, the samples of interest for a study project are chosen and an appropriate amount of the selected DNAs is withdrawn from the archive (hit picking) and stored in working plates. For efficient and reliable processing of a large number of samples, a pipeting robot is required. Normalization, archivation and hit picking are performed by means of a robot as well as the pipeting of SNP discrimination reactions. All samples and microtiter plates have to be barcoded for the fast manual or automated identification of samples and plates and for the recording of performed work steps. Automated data processing and sample tracking during the whole workflow guarantees ready availability of test results subsequent to assignment of genotypes to the obtained test results (genotyping).

1. **Isolation of DNA**
2. **Determination of concentration and normalization**
3. **Archivation**
4. **Hit picking**
5. **Pipeting of SNP reaction**
6. **Genotyping**

Tab. 5.8
High-throughput workflow process.

The pipeting robot Biomek FX (Beckman Coulter, Fullerton, CA, USA), equipped with an 8-channel pipettor and a 96-channel pipettor, was used for this work. The 8-channel pipettor, with independent volume control of each channel, is ideal for transferring individual samples into the 96-well format (archivation), for individual dilution of samples (normalization) and for picking single samples out of a microtiter plate (hit picking). The 96-channel pipettor is best suited for transferring material from one whole plate into another (e.g. adding DNA to the reaction plate). In addition, the Biomek FX contains a barcode reader and a stacker carousel that can hold additional 40 plates or tip boxes to be used with the robot, which both are essential for a high-throughput workflow.

For normalization and archivation, barcoded DNA samples were transferred to deep-well-microtiter-plates using the pipeting robot. Up to five identical plates were generated with 50 µl of original DNA eluate each. The position of DNA samples in the microtiter-plates were stored together with their barcodes in a SYBASE database. To determine the concentration of DNA, a PicoGreen assay (Molecular Probes, Eugene, OR, USA) was performed. One microliter of DNA was transferred to PicoGreen assay reagent with the 96-channel pipettor. Using Fluostar BMG-Reader, connected to Biomek FX software, DNA concentration was determined. Then BFX normalization software provided from Beckman Coulter calculated the amount of water to be added to each well, to dilute DNA samples to a uniform concentration of 10 ng/µl in the deep well plates. BFX normalization software generated a working list for Biomek FX Pipeting Robot and dilution was carried out automatically. The normalized DNA Bank was stored at -80°C.

Cohorts were constituted by hit picking from probands sharing similar clinical phenotypes such as premature coronary artery disease or diabetes mellitus, or aberrant lipid profiles. With custom made storage management program, all necessary

barcoded plates of the DNA-bank were acquired and transferred to the Biomek FX Stacker Carrousel. DNA of the relevant probands was picked from the normalized DNA-bank automatically using Biomek FX Hit Picking Wizard. Usually, 96-well (round-bottom) working plates were generated that contained up to 200 µl of DNA each at a concentration of 5 ng/µl.

Assays were carried out in a total volume of 5 µl. With Biomek FX, using 96-multichannel pipettor, 4 µl of master mix, containing probes and primers, was transferred to each well of a 384-well-reaction plate. Then DNA from four 96-well round-bottom working plates was added (1 µl each). Reaction plates were sealed with a heat sealer (Abgene, Epsom, UK) and cycled on MWG Primus multiblock thermo-cyclers (MWG, Ebersberg, Germany). Endpoint fluorescence measurement was carried out on TaqMan 7900HT using Zymark Twister for automated plate loading.

High-throughput SNP genotyping requires sophisticated software infrastructure. Using SYBASE database (Sybase, Dublin, CA, USA) and custom made C^{++} programs (Borland Software Corporation, Scotts Valley, CA, USA), sample tracking, hit picking, DNA storage management and automated result generation were achieved. In Fig. 5.7, data handling in the high-throughput workflow is schematically illustrated. Patients and DNA samples (more than one sample can belong to a single patient) are fed into the database. Numbers and barcodes are assigned for patient and sample automatically and linked to each other. The barcoded DNA samples are transferred to storage plates and the barcode of the storage plate, the assignment of the well position and the archived amount of samples is stored. For hit picking, the relevant storage plates are selected from the database. The amount of DNA withdrawn is subtracted from the storage plate entry, the barcode and the assignment of the well positions are fed into the database. The database also contains all relevant assay information, like name and assignment of fluorescent signal and genotype. Then, for TaqMan analysis, only barcodes of the working plates and the reaction plate have to be scanned and the performed assay has to be named. Results are generated in tabular form automatically, after the operator has gated the raw data.

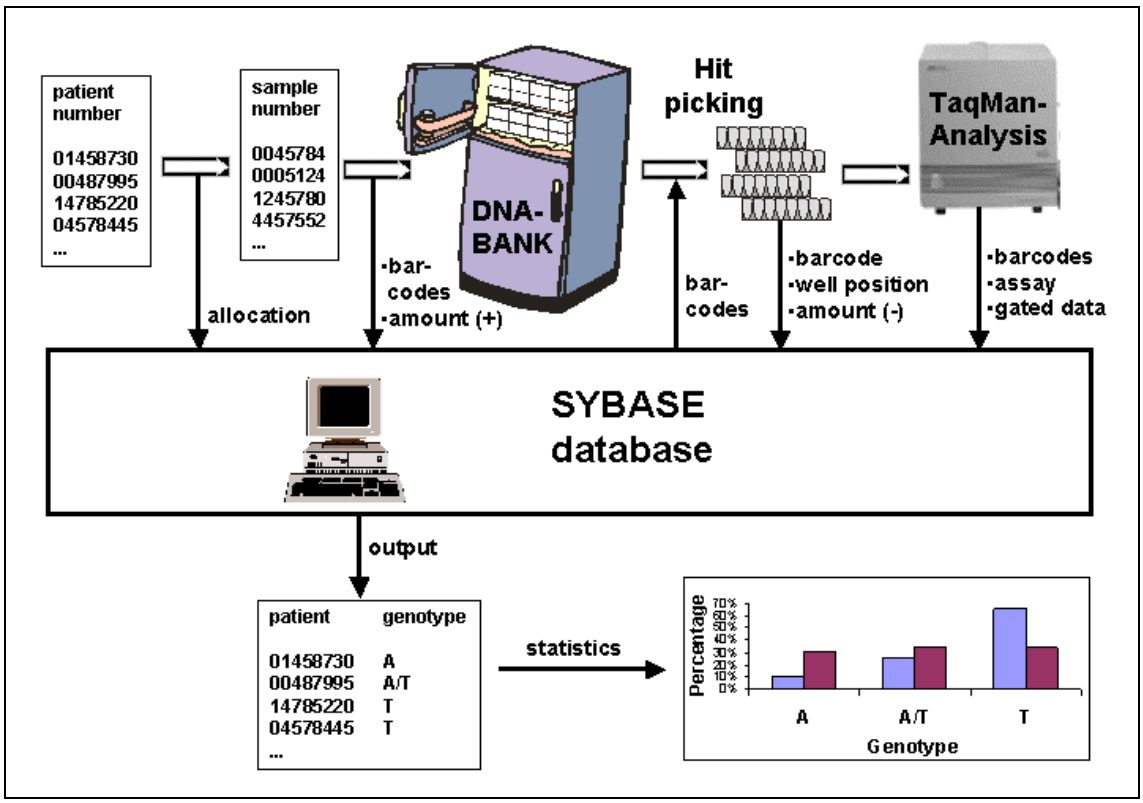

Fig. 5.7
Schematic representation of data management in high-throughput workflow.

5.2.4 Results of genotyping

In the main focus of genotyping was the novel polymorphism V771M, which has not yet been characterized further. The relevant polymorphisms R219K, I883M and E1172D were genotyped as well, when DNA material was not limited. Very little material of the Hungarian and Italian cohorts was available, so that not all polymorphisms could be investigated in these cohorts.

A significant difference in genotype distribution was not detected in healthy blood donors from three different ethnical origins (Tab. 5.9). A lower prevalence of K219 homozygotes in Italian donors (2.7%) and an increased amount of K219 homozygotes in Hungarians (11.9%) compared to Germans (7.4%) was found. It should also be stated, that Hardy Weinberg disequilibrium (p_{HWE}=0.017*) was detected in the Italian donors cohort for the R219K polymorphism. The M771 allele was more abundant in Italians (8.9% heterozygotes) and Hungarians (9.8% heterozygotes) compared to Germans (5.6% heterozygotes). However, these differences are not significant (p=0.125 and 0.052, respectively). M771 homozygotes were not found.

No significant differences in genotype distribution were also found in comparison of all German cohorts (Tab. 5.10). A slightly higher prevalence of the I883 allele in the diabetes cohort (79.8% homozygotes) compared to healthy blood-donors (75.6%) was detected (p=0.110). All genotype distributions were in Hardy Weinberg equilibrium.

In the Hungarian cohorts (Tab. 5.11), the V771 homozygotes were more prevalent in CAD patients (97.3%) compared to healthy blood donors (90.2%). The difference was highly significant (p=0.008**). This could indicate, that the M771 variant has a protective effect in the development of CAD. Homozygotes for V771 were also more frequent in the stroke cohort (94.3%), but this did not reach significance (p=0.133).

In Italian centenarians (Tab. 5.12), the number of V/M771 heterozygotes was slightly increased (12.7%) compared to Italian blood donors (8.9%), however not significantly. Therefore, R/K219 heterozygotes were significantly more abundant (p=0.016*) in centenarians (46%) compared to the Italian control group (32.4%). In DON_I 48.7% were carriers of the K219 allele, in CENT 41.4%.

Further (advanced) statistical analysis of the genotyping data was not performed, since in large German cohorts no association was obvious and splitting up the Italian and Hungarian cohorts into subgroups (age, sex) would be problematic due to the low number of individuals. The aim of this work was not to exhaust statistical data analysis methods to find weak associations, but to set up functional assays in the high-throughput format.

R219K	**N**	**R**	**R/K**	**K**	p_{HWE}	$p(\chi^2)$
DON	458	226 (49.3%)	198 (43.2%)	34 (7.4%)	0.364	–
DON_H	193	97 (50.3%)	73 (37.8%)	23 (11.9%)	0.128	0.128
DON_I	150	77 (51.3%)	69 (46.0%)	4 (2.7%)	**0.017***	0.112
V771M	**N**	**V**	**V/M**	**M**	p_{HWE}	$p(\chi^2)$
DON	478	451 (94.4%)	27 (5.6%)	0 (0.0%)	1.000	–
DON_H	193	174 (90.2%)	19 (9.8%)	0 (0.0%)	1.000	0.052
DON_I	191	174 (91.1%)	17 (8.9%)	0 (0.0%)	1.000	0.125
I883M	**N**	**I**	**I/M**	**M**	p_{HWE}	$p(\chi^2)$
DON	467	353 (75.6%)	105 (22.5%)	9 (1.9%)	0.686	–
DON_H	105	80 (76.2%)	24 (22.9%)	1 (1.0%)	1.000	0.788
DON_I	191	140 (73.3%)	49 (25.7%)	2 (1.0%)	0.541	0.521
E1172D	**N**	**E**	**E/D**	**D**	p_{HWE}	$p(\chi^2)$
DON	478	456 (95.4%)	22 (4.6%)	0 (0.0%)	1.000	–
DON_I	190	177 (93.2%)	12 (6.3%)	1 (0.5%)	0.220	0.185

Tab. 5.9
Distribution of genotypes in control groups (healthy blood donors) from three different ethnical origins (Germans, Hungarians and Italians). M771 seems to be more prevalent in Hungarians and Italians. The R219K polymorphism was in Hardy Weinberg disequilibrium in Italian donors. N, number of individuals.

R219K	N	R	R/K	K	p_{HWE}	$p(\chi^2)$
DON	458	226 (49.3%)	198 (43.2%)	34 (7.4%)	0.364	–
TTDM	375	191 (50.9%)	154 (41.1%)	30 (8.0%)	1.000	0.810
OCT	434	224 (51.6%)	170 (39.2%)	40 (9.2%)	0.350	0.371
CAD	613	333 (54.3%)	243 (39.6%)	37 (6.0%)	0.461	0.243
V771M	**N**	**V**	**V/M**	**M**	p_{HWE}	$p(\chi^2)$
DON	478	451 (94.4%)	27 (5.6%)	0 (0.0%)	1.000	–
TTDM	376	359 (95.5%)	17 (4.5%)	0 (0.0%)	1.000	0.459
OCT	422	394 (93.4%)	27 (6.4%)	1 (0.2%)	0.392	0.505
CAD	614	582 (94.8%)	31 (5.0%)	1 (0.2%)	0.357	0.617
I883M	**N**	**I**	**I/M**	**M**	p_{HWE}	$p(\chi^2)$
DON	467	353 (75.6%)	105 (22.5%)	9 (1.9%)	0.686	–
TTDM	377	301 (79.8%)	74 (19.6%)	2 (0.5%)	0.403	0.110
OCT	434	344 (79.3%)	85 (19.6%)	5 (1.2%)	1.000	0.340
CAD	611	452 (74.0%)	146 (23.9%)	13 (2.1%)	0.783	0.831
E1172D	**N**	**E**	**E/D**	**D**	p_{HWE}	$p(\chi^2)$
DON	478	456 (95.4%)	22 (4.6%)	0 (0.0%)	0.781	–
OCT	434	417 (96.1%)	17 (3.9%)	0 (0.0%)	1.000	0.609
CAD	614	585 (95.3%)	29 (4.7%)	0 (0.0%)	1.000	0.925

Tab. 5.10
Distribution of genotypes in German cohorts. No significant differences were found. DON, donors; TTDM, diabetes; OCT octogenarians.

R219K	N	R	R/K	K	p_{HWE}	$p(\chi^2)$
DON_H	193	97 50.3%	73 37.8%	23 11.9%	0.128	–
STROKE	247	134 54.3%	84 34.0%	29 11.7%	0.008	0.679
CAD_H	152	84 55.3%	57 37.5%	11 7.2%	0.833	0.317
V771M	**N**	**V**	**V/M**	**M**	p_{HWE}	$p(\chi^2)$
DON_H	193	174 90.2%	19 9.8%	0 0.0%	1.000	–
STROKE	245	231 94.3%	13 5.3%	1 0.4%	0.198	0.133
CAD_H	150	146 97.3%	4 2.7%	0 0.0%	1.000	**0.008****
I883M	**N**	**I**	**I/M**	**M**	p_{HWE}	$p(\chi^2)$
DON_H	105	80 76.2%	24 22.9%	1 1.0%	1.000	–
STROKE	246	178 72.4%	61 24.8%	7 2.8%	0.466	0.492
CAD_H	153	120 78.4%	29 19.0%	4 2.6%	0.238	0.499

Tab. 5.11
Distribution of genotypes in Hungarian cohorts. V771 homozygotes were more prevalent in CAD patients (97.3%) compared to healthy blood donors (90.2%). The difference was highly significant (p=0.008**). This could indicate, that the M771 variant has a protective effect in the development of CAD. Homozygotes for V771 were also more frequent in the stroke cohort (94.3%), but this did not reach significance (p=0.133).

R219K	N	R	R/K	K	p_{HWE}	$p(\chi^2)$
DON_I	150	77 51.3%	69 46.0%	4 2.7%	**0.017***	–
CENT	111	65 58.6%	36 32.4%	10 9.0%	0.136	**0.016***
V771M	**N**	**V**	**V/M**	**M**	p_{HWE}	$p(\chi^2)$
DON_I	191	174 91.1%	17 8.9%	0 0.0%	1.000	–
CENT	126	110 87.3%	16 12.7%	0 0.0%	1.000	0.279
I883M	**N**	**I**	**I/M**	**M**	p_{HWE}	$p(\chi^2)$
DON_I	191	140 73.3%	49 25.7%	2 1.0%	0.541	–
CENT	104	72 69.2%	31 29.8%	1 1.0%	0.458	0.745
E1172D	**N**	**E**	**E/D**	**D**	p_{HWE}	$p(\chi^2)$
DON_I	190	177 93.2%	12 6.3%	1 0.5%	0.220	–
CENT	118	109 92.4%	9 7.6%	0 0.0%	1.000	0.667

Tab. 5.12
Distribution of genotypes in Italian cohorts. The number of V/M771 heterozygotes was slightly increased (12.7%) in centenarians (CENT) compared to healthy blood donors (8.9%), however not significantly. Therefore, R/K219 heterozygotes were significantly more abundant (p=0.016*) in centenarians (46%) compared to the Italian control group (32.4%).

5.3 Development of a bead-based multiplex assay

Four SNPs resulting in amino acid exchanges in the ABCA1 protein product that have shown association, have been described: R219K, V771M, I883M and E1172D. In this part, a multiplex panel will be presented, that allows the simultaneous detection of all four SNPs. This panel uses the new bead-based Luminex technology described in chapter 2. The motivation of this work was to evaluate the Luminex platform for the determination of SNP risk profiles and to compare this method to other established technologies, especially the LightCycler and the TaqMan technology.

Several formats of wetware have been tested to establish a functional assay panel for the Luminex technology, including competitive hybridization, minisequencing (SBCE) and allele specific primer extension (ASPE). Competitive hybridization was found to be less straightforward. Multiple sequences, partly very similar to each other need to be optimized for identical hybridization conditions and high specificity and sensitivity at the same time. Although it is possible to develop assays using this technique, which has been shown (see 2.4.3.2), intensive optimization is required. This was noticed at an early stage of development and the method was rejected. Instead, a set of functional minisequencing primers was developed, which worked well with ABI Prism Snap Shot (Applied Biosystems, Foster City, USA) technology using a capillary sequencer (data not shown). However, the primers were not adapted to bead-based analysis, since up to four separate reactions would have been required or a more sophisticated flow cytometer (capable of reading six fluorescent channels) would have been necessary (see 2.4.3.3). Instead, the allele specific primer extension method was used to develop a functional assay, which is very well suited for the Luminex platform (see 2.4.3.3 and Fig. 2.16).

5.3.1 Design of primers

PCR primers were designed using primer3 software.[99] The relevant segments of DNA to be amplified and the location of the sequence variation therein (green color) are presented in Fig. 5.8. The designed primers are marked in blue color. To obtain similar amplification efficiency for all amplicons, primers were designed to give product sizes between 150 and 200 base pairs, with at least 10 base pairs in difference to allow separation by agarose gel electrophoresis. Similar melting temperatures of primers were aimed at, between 55 and 60°C. Primer sequences, melting points and amplicon sizes are listed in Tab. 5.13. Primers were checked for cross-hybridization to other genes by blast-search [100] and to repeat regions using primer3 mispriming library.[101]

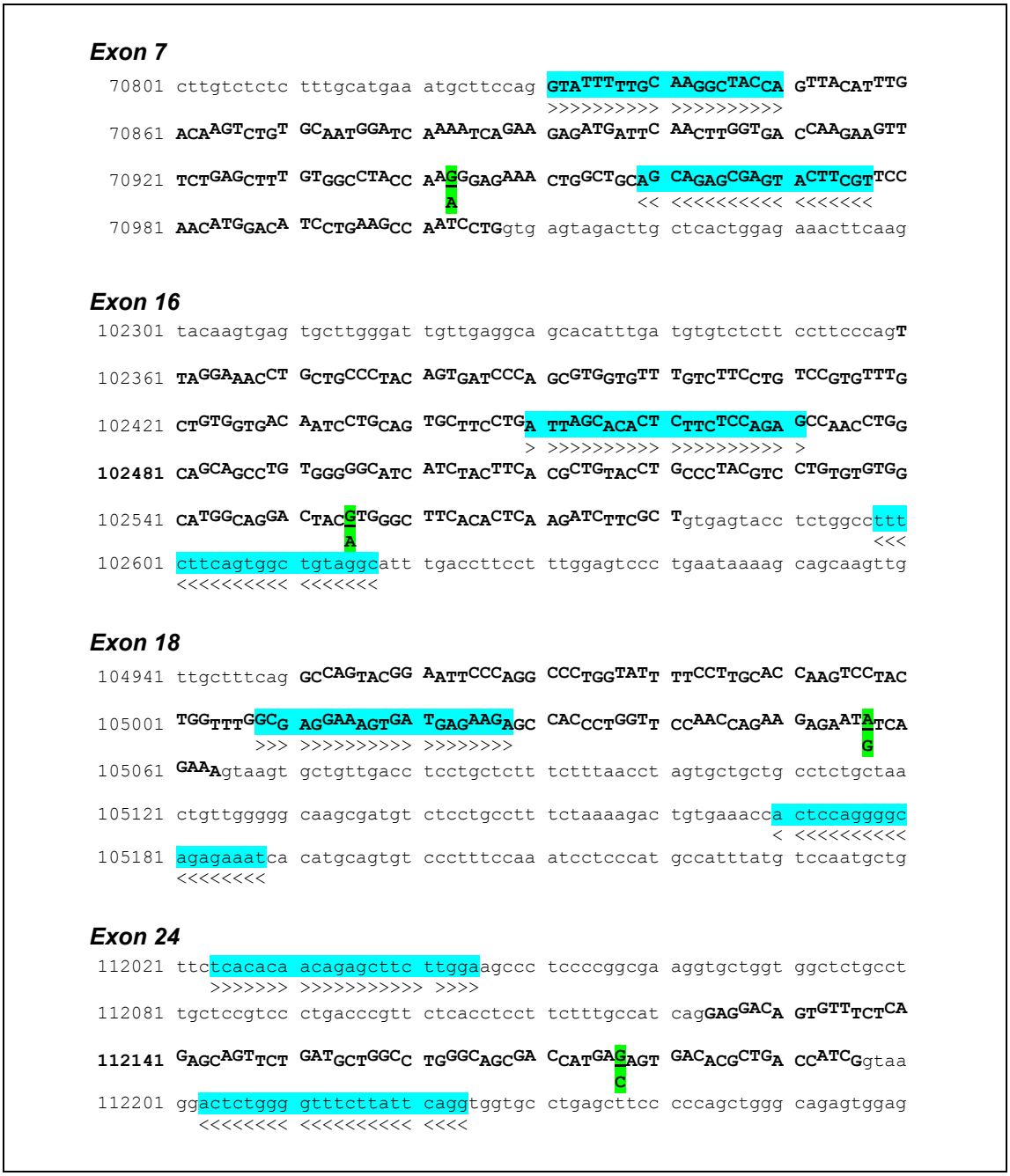

Fig. 5.8
Exon 7 containing R219K (G70943A), Exon 16 containing V771M (G102555A), Exon 18 containing I883M (A105057G), Exon 24 containing E1172D (G112177C). Primers are marked in blue, the SNP in green color. Codons are printed alternately in superscript and normal bold letters. Intronic sequence is printed in lower case letters.

5.3.2 Multiplex-PCR

A multiplex PCR was performed using Hot Star Kit (Qiagen, Hilden, Germany). In initial experiments, week bands of amplicons were improved by increasing the amount of used primer. Optimum primer concentration were: R219K and E1172D 0.2 µM each, V771M 0.3 µM each and I883 0.8 µM each. Salt concentrations were also varied in initial experiments and the reaction was performed with and without additiva (Q-solution). Optimum results were gained with the reaction mix containing dNTPs 0.2 mM

each, 3 mM $MgCl_2$, 2x PCR Buffer, 1x Q-solution and 0.05 U/µl Hot Star polymerase, as well as approximately 4 ng/µl genomic DNA. The reaction mixture was heated at 95°C for 15 minutes, then 40 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 30 seconds at 72°C were performed. For final extension, the mixture was incubated at 72°C for 7 minutes. A PTC-200 thermal cycler (MJ Research, Waltham, MA, USA) was used. For amplification of single PCR products for initial experiments in allelic discrimination, only one pair of primers was used with the same concentrations and conditions as in the multiplex PCR. Fig. 5.9 shows agarose gel electrophoresis of single and multiplex PCR products.

Primer	Sequence	Tm	Position	Amplicon Size
R219K-F	GTA TTT TTG CAA GGC TAC CA	55.2°C	70,831-70,850	147 bp
R219K-R	ACG AAG TAC TCG CTC TGC T	55.0°C	70,977-70,959	
V771M-F	ATT AGC ACA CTC TTC TCC AGA G	55.1°C	102,450-102,471	168 bp
V771M-R	GCC TAC AGC CAC TGA AGA AA	57.8°C	102,617-102,598	
I883M-F	GCG AGG AAA GTG ATG AGA AGA	59.3°C	105,008-105,028	181 bp
I883M-R	ATT TCT CTG CCC CTG GAG T	58.4°C	105,188-105,170	
E1172D-F	TCA CAC AAC AGA GCT TCT TGG A	60.8°C	112,024-112,045	201 bp
E1172D-R	CCT GAA TAA GAA ACC CCA GAG T	58.4°C	112,224-112,203	

Tab. 5.13
Primers for (multiplex) PCR.

5.3.3 Allelic discrimination

ASPE-primer were designed manually, with melting-points of the sequence specific 3'-residue of 55°C to 60°C. Different lengths of primers within that range of melting temperature and hybridizing to both, sense or antisense strand, were tested to function with purified single and multiplex PCR product. Always a pair of primers, targeting both forms of one sequence variation at the same strand, were tested. This ensures minimum cross-talk due to competition of ASPE-primers. Control DNAs with all possible alleles in homozygous and heterozygous form were selected from the collection of genotyped samples presented in 5.2 and confirmed by DNA sequencing. All possible primer combinations were tested and evaluated with respect to maximum signal intensity, minimum cross-talk (signal intensity of false allele) and minimum background (signal without PCR product). The best set of ASPE primers is presented in Tab. 5.14. The zip-code sequences for the primer set were selected using Tag IT Oligo Design Software [102] with respect to minimum cross-hybridization of zip codes

and primers. The sequences of the zip codes are abbreviated by LUA (LUminex Array) plus the corresponding number of zip code (e.g. LUA19 refers to zip code number 19). The number of the zip code is identical with the number of FlexMAP Bead, carrying the reverse complement of the zip-code sequence. All zip codes are listed in Tab. 3.1 of the appendix.

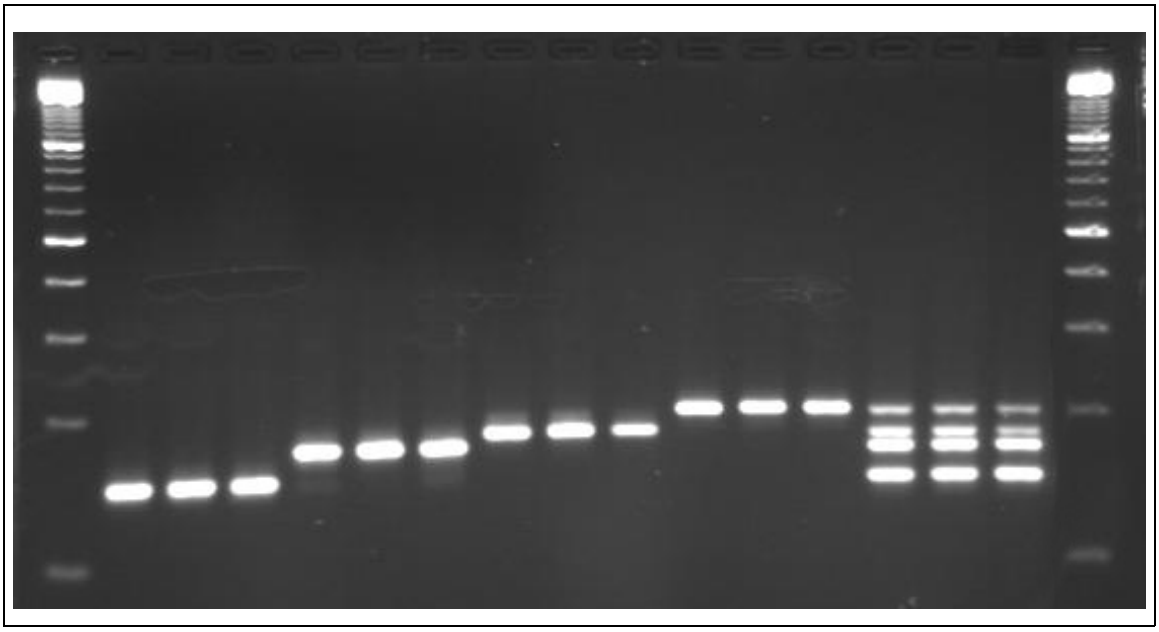

Fig. 5.9
Single and multiplex-PCR products of 3 probands, containing polymorphic loci R219K (147 bp), V771M (168 bp), I883M (181 bp) and E1172D (201 bp), separated on a 3% agarose gel.

Primer	Sequence	T_m of sequence specific 3'-moiety
R219	LUA19-AGCTTTGTGGCCTACCAA**G**	57.7°C
K219	LUA08-GCTTTGTGGCCTACCAA**A**	56.9°C
V771	LUA72-AGATCTTGAGTGTGAAGCCCA**C**	60.5°C
M771	LUA07-AGATCTTGAGTGTGAAGCCCA**T**	59.9°C
I883	LUA28-TGGTTCCAACCAGAAGAGAAT**A**	58.5°C
M883	LUA99-GGTTCCAACCAGAAGAGAAT**G**	58.4°C
E1172	LUA17-GATGGTCAGCGTGTCACT**C**	57.3°C
D1172	LUA29-GATGGTCAGCGTGTCACT**G**	58.4°C

Tab. 5.14
ASPE primer carrying a unique zip-code at their 5'-end. The number of zip-code refers to Tab. 3.1.

The PCR product (10 µl) was treated with a mixture of shrimp alkaline phosphatase (SAP) and exonuclease I to remove unincorporated dNTPs and excess primers, respectively. The purification mix (10 µl), which consists of 2 units of SAP (Roche Applied Sciences, Penzberg, Germany) and 1 unit of exonuclease I (Amersham Biosciences, NJ, USA) in 1x phosphatase buffer (Roche Applied Sciences, Penzberg, Germany), was added to the PCR-product and the mixture was incubated at 37°C for 45 minutes, then kept at 95°C for 15 minutes to inactivate the enzymes.

Allele specific primer extension was performed using ASPE-primers (Tab. 5.14) carrying Luminex zip code sequences. HPSF purified oligonucleotides were purchased from MWG Biotech (Ebersberg, Germany). A 20 µl reaction contained 5 µl of treated PCR product, 1.25 mM $MgCl_2$, 5 µM each dNTP, 5 µM biotin-dCTP (Perkin Elmer Life Sciences, Boston, MA, USA), 25 nM each primer and 0.075 U/µl Platinum GenoTYPE Tsp polymerase in 1x Tsp buffer (Invitrogen, Carlsbad, CA, USA). In a PTC-200 thermocycler (MJ Research, Waltham, MA, USA), after two minutes initial denaturation at 95°C, 40 cycles of 30 seconds at 94°C, 30 seconds at 54°C and 60 seconds at 74°C were performed.

Hybridization of ASPE product to FlexMap beads was carried out in Tm buffer (2x Tm buffer contains 0.2M NaCl, 0.1M TRIS and 0.08% Triton X-100 at pH 8.0). The ASPE product (5 µl) was incubated with 2,500 LUA beads of each region in in a total volume of 50 µl 1x Tm buffer (0.2M NaCl, 0.1M TRIS and 0.08% Triton X-100 at pH 8.0) at 96°C for 90 seconds, then kept at 37°C for 60 minutes. The microtiter-plate was centrifuged at 3,000 rcf for 2 minutes, supernatant was removed and 65 µl of streptavidin-PE conjugate (Molecular Probes, Oregon, USA) in a concentration of 2 µg/ml in 1x Tm buffer were added. The mixture was incubated at 37°C for 15 minutes.

Samples were measured on the Luminex 100 at 37°C. Acquisition of 600 beads total with a timeout of 60 seconds and a gating of 7,500-15,000 units were selected. Median fluorescence intensities were recorded.

For data evaluation, background signals from the negative control (no template in PCR) were subtracted from recorded fluorescence intensities. An assay that did not produce a signal intensity of 200 a.u. above the background in both alleles is neglected for the individual sample (dropout). For better visualization and improved genotyping, fluorescence intensities were normalized to total fluorescence intensity of both alleles; the cutoff for allelic discrimination was set to 0.25 units (Fig. 5.10).

To evaluate the developed multiplex assay, a total of 200 DNA samples were analyzed with the new method as described above and compared to TaqMan assays, which were performed in parallel. For TaqMan analysis, the set of primers and probes presented in 5.2 were used.

For evaluation, the percentage of dropouts for each method and the concordance of both methods were determined. In order to assess, whether the number of dropouts was significantly different in both methods, McNemar test (χ^2 test for matched pair data) was applied. If $p_{McNemar}$ was smaller than 0.05, significantly more dropouts were produced in one method. Confidence intervals for concordance were calculated at a 95% confidence level by the Clopper and Pearson method.[103]

5.3.4 Genotyping results using the multiplex bead assay

The results obtained by the Luminex method were plotted in discrimination plots only for better visualization. Genotyping was performed in Excel spreadsheets using the cutoffs described above. In these plots, the normalized fluorescence intensities of each probe are plotted against each other and cutoffs (0.25 units) are drawn in. The dropouts (not producing a significant signal above the background, cutoff 200 a.u.) were removed before assignment of genotypes. In the discrimination plots in Fig. 5.10, the four different populations (including no template controls) can clearly be identified.

To assess analytical sensitivity, McNemar test was applied to compare the assays for both platforms with respect to dropouts and good signals only (Tab. 5.15). A significant difference was found in assays for R219K (p=0.002**) and E1172D (p=0.000***). Judging from the amount of dropouts in both assays and assuming that the remaining analyses produced correct results, it seems that the Luminex assay for R219K might be more sensitive compared to the TaqMan assay, however the Luminex assay for E1172D appears to be less sensitive. The assays for V771M and I883M seem to be comparable.

By looking solely at data that had been assigned by both methods, concordances were calculated. Within a 95% confidence interval, concordances of above 95% (lower limit of confidence interval) were obtained for all assays (Tab. 5.16). Altogether, five SNP determinations showed discrepancy. These samples were further analyzed by DNA sequencing (Tab. 5.17) and it was found that 3 samples were falsely assigned by the TaqMan method and two by the Luminex method. Assuming, that concordant samples have been genotyped correctly, the accuracy of the newly developed Luminex assays in this evaluation were 100% for R219K and I883M, 99.4% for V771M and 99.2 for E1172D.

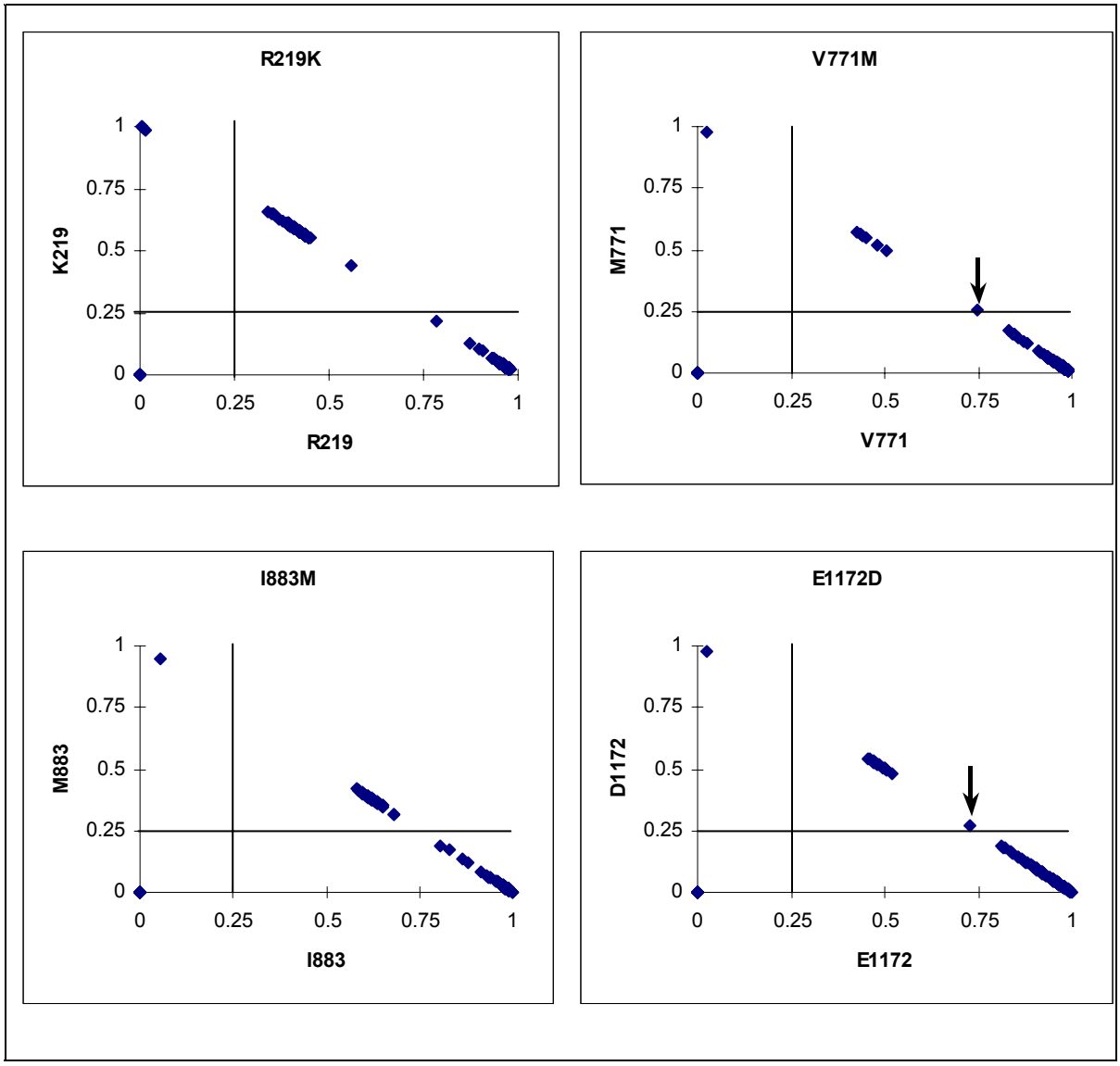

Fig. 5.10
Discrimination plots for Luminex assays R219K, V771M, I883M and E1172D. Normalized fluorescence intensities of both probes are plotted against each other. The cutoff for allelic discrimination was set to 0.25 units. Wrongly assigned samples are marked with an arrow.

	TaqMan		Luminex		$p_{McNemar}$
R219K	30	(15%)	12	(6%)	**0.002****
V771M	15	(7.5%)	21	(10.5%)	0.286
I883M	29	(14.5%)	23	(11.5%)	0.392
E1172D	30	(15%)	56	(28%)	**0.000*****
overall	104	(13%)	112	(14%)	0.554

Tab. 5.15
Amount of dropouts in both methods for 200 DNA samples. The Luminex assay for R219K seems to be more sensitive compared to the TaqMan assay, however the Luminex assay for E1172D appears to be less sensitive.

	concordant	discrepant	concordance	95% CI
R219K	161	2	98.8%	0.956-0.999
V771M	170	1	99.4%	0.967-1.000
I883M	156	1	99.4%	0.965-1.000
E1172D	130	1	99.2%	0.958-1.000
overall	617	5	99.2%	0.981-0.997

Tab. 5.16
Concordance of genotyping-results (dropouts neglected). Within a 95% confidence interval, concordances of above 95% (lower limit of confidence interval) were obtained for all assays.

	total discrepant	false TaqMan	false Luminex	accuracy Luminex
R219K	2	2	0	100.0%
V771M	1	0	1	99.4%
I883M	1	1	0	100.0%
E1172D	1	0	1	99.2%
overall	6	3	2	99.5%

Tab. 5.17
Number of false results as confirmed by DNA sequencing of discrepant samples. Assuming, that concordant samples have been genotyped correctly, the accuracy of the newly developed Luminex assays are 99.2% and above.

6 Discussion

6.1 Screening for functional sequence variations and mutations in ABCA1

In previous studies, the ABCA1 promoter region was characterized and transcription factor binding sites were identified (see 2.1.5). Variable regulation of the ABCA1 expression will ultimately lead to different levels of susceptibility to HDL levels, CVD and other ABCA1 related phenotypes. Therefore, the investigation of the promoter region is of great importance. Sequence variations in the promoter region, especially at transcription factor binding site might influence binding of transcription factors and hence the expression of the ABCA1 gene.

The screening that has been performed of the promoter region was originally not intended to be an association study. The main focus was the discovery of novel sequence variations therefore the number of individuals investigated was low. However good levels of significance were obtained and that the number of individuals that have been sequenced is not too low compared to other high-throughput association studies is supported by the fact, that the results are coherent with the findings of two other groups. The G allele of C1176G in the promoter (Fig. 5.1) was previously associated with CAD [89] and it was found significantly decreased in the High-HDL cohort compared to DON and the Low-HDL group. The G allele of G1355C (Fig. 5.1) was earlier associated with decreased CAD [90], what is conclusive with the finding, that G1176 is significantly more prevalent in High-HDL compared to Low-HDL, DON and Octogenarians. The three novel polymorphisms (G1047C, C1152T and C1440T) were found in significantly different distribution of genotypes in some cohorts (Tab. 5.1). All three exchanges (especially heterozygotes) were found more frequent in probands of the Low-HDL cohort compared to other groups. Therefore, it can be assumed, that these three polymorphisms are associated with decreased HDL levels. However, further functional testing and haplotype analysis in larger cohorts might be required to fully confirm this.

The VNTR-ZNF that was identified is part of the ZNF202 transcription factor binding-site. ZNF202 is known to down-regulate the expression of ABCA1 and many other genes involved in cholesterol metabolism, therefore this variation was investigated further. Although there was evidence for different binding of ZNF202 in vitro, the in vivo assay did not display a significant effect of this promoter polymorphism. The two VNTR polymorphisms might be due to ethnical differences and possibly do not influence ABCA1 expression. The shorter form of the VNTR-ZNF polymorphism (ΔACCCC) was

always found in conjunction with the VNTR-SRY wild-type form, which is good evidence, that VNTR-ZNF (ΔACCCC) is coupled to VNTR-SRY wild-type form. The wild-type form of VNTR-ZNF was found with all forms of VNTR-SRY polymorphisms. The VNTR-ZNF wild-type and the shorter form of VNTR-SRY (ΔGTTTTGTTT) seem to be most prevalent in European Caucasians (60% and 49%, respectively; see Tab. 5.2 and Tab. 5.3).

Sequencing of exons 49 and 50 revealed only one sequence variation in 249 individuals. The fact, that there are almost no polymorphic loci in this region could stress the importance of the carboxyterminus of ABCA1, which is coherent with the phenomenon of protein-protein interaction observed in this particular region. Nonetheless, this region is very small (385 bp) and therefore not many sequence variations are expected to be found. However, the sequence variations found in patient E with G6PD deficiency (F2163S, V2244I) are located in exon 49 and 50, respectively and these mutations may account for the low HDL-cholesterol. This is supported by the fact that one of the enzymes that was identified in the yeast two hybrid system to interact with the COOH-terminus of ABCA1 is part of the succinate dehydrogenase complex (iron sulfur complex) [45], which is known to be involved in the pathogenesis of hypertrophic cardiomyopathy. The coincidence of polymorphisms within the carboxyterminus of the ABCA1 gene and clinical correlates of succinate dehydrogenase deficiency, hypertrophic cardiomyopathy and skeletal muscle myopathy in the father (pedigree C, Fig. 3.1) without mutations in the G6PD-gene indicates that the mutation in exon 50 of ABCA1 is causally related to dysfunctions of succinate dehydrogenase and/or G6PD, perhaps by interfering with correct protein-protein interactions and failure of shuttling between ABCA1 and the inner mitochondrial membrane.

If the introduced methionine at codon 771 is responsible for the increased HDL levels of patient A remains unclear. But since no sequence variation in CETP, SR-BI and the LDL receptor was found in this individual, and V/M771 heterozygote family members had lower HDL levels, it might seem conceivable that this sequence variation had an effect on HDL levels. In addition, methionine homozygotes seem to be extremely rare, which was confirmed by genotyping of 478 blood donors, in which none was found. It could be speculated, that the introduced methionine at codon 771 could be of regulatory importance for ABCA1 through the oxidation of methionine (methionine sulfoxide) by various reactive oxygen species (ROS). In lipoprotein metabolism (cholesterol efflux) it is known that methionine oxidation in apo A-I is a key regulatory event in LCAT activation [104], thus the amino acid exchange valine to methionine at codon 771 may affect functionality.[105-107] Moreover, V771M is located very close to a putative transmembrane region (Fig. 5.3) and it is known that mutations in the SUR1

gene that are localized near the transmembrane region impair the interaction of this ABC transporter with Kir6.2 channel.[108] Therefore, it can be speculated that the V711M polymorphism in ABCA1 may affect interaction with associated proteins or ABCA1 function. It is also possible, that the patient has an additional mutation in such an interacting protein.

In the probands with HDL deficiency (patient B, C and D) three novel sequence variations have been identified: W840R, W590L and R1068C. It is very likely, that amino acid exchange W840R in the 50-year old female (patient B) is an additional Tangier mutation. The sequence variation was not found in a large cohort, which indicates, that it is not a polymorphism. It can also be assumed, that amino acid exchange W590L, found in patient C, is a mutation causing a defective ABCA1 protein product. This is supported by the fact, that a mutation in the same codon was shown to cause Tangier Disease (W590S) [17], but since the patient is only heterozygous for W590L, it remains unclear, what additional mutation in ABCA1 causes the patients analphalipoproteinemia. It is very likely, that the amino acid exchange R1068C, encountered in patient D, results in a defective or less active ABCA1 product. Although the mother, who is heterozygous for this sequence variation, was found to have quite normal HDL and apo A-I levels, the index patient who is compound heterozygous for R1068C and a known Tangier mutation (ΔTC in exon 23), showed analpha-lipoproteinemia. The causative effect of the mutation is supported by the fact, that the affected amino acid is located very close to walker B of the first ATP-binding cassette, which is a highly conserved region (Fig. 5.3). The father and the sister, who are heterozygous for the Tangier mutation and have no additional R1068C mutation, show typical reduced HDL levels but no analphalipoproteinemia.

This study has revealed a number of novel sequence variations. Three SNPs in the promoter were associated with low HDL levels and one VNTR polymorphism indicated different binding of transcription factor ZNF202. Three novel mutations have been identified in patients with HDL deficiency. Two mutations have been found in a phenotype related to CVD, located in an important domain of ABCA1 (carboxy-terminus) and a rare homozygous polymorphism (V771M) was identified in a proband with extremely high HDL.

Although ABCA1 is known to play a key role in reverse cholesterol transport and several mutations in this gene have been identified to cause HDL deficiency syndromes, the regulation and activity of ABCA1 and the influence of sequence variations in these processes are still very limited. Further research is required in this area. For this reason, a webpage archive for sequence variations in ABCA1 has been created (http://www.abca1-mutants.all.at). The aim of this site is to store sequence variation relevant data and epicrisis of affected individuals. This should help to

elucidate important domains in ABCA1 and help in understanding function and role of ABCA1 in regulating plasma HDL concentration. This webpage provides a more recent and detailed summary of sequence variations in ABCA1 than existing databases and should be of interest for further research in ABCA1 and molecular diagnosis of ABCA1 related HDL deficiency.

6.2 High-throughput genotyping of ABCA1 polymorphisms

The TaqMan (ABI Prism 7900HT) is very well suited for automation, since it applies standard microtiter plates, which can be handled routinely in roboting platforms. For the LightCycler a round shaped sample holder (carrousel) is required, that cannot be used with common robots. Although, with the MagNa Pure LC, a robot exists for the isolation of DNA from low volume samples that can as well handle LightCycler carrousels, the loading of the carrousel with fragile capillaries prior to pipeting and the capping of the capillaries afterwards still has to be done manually. The maximum capacity of a LightCycler carrousel is 32 samples, while with TaqMan 384-well plates are processed. In addition, reaction plates do not have to be thermally cycled in the TaqMan, additional standard thermal cyclers without fluorescence unit can be used to liberate bottle-neck capacity. Also, analytical cost can be cut. In the LightCycler, a total assay volume of 20 µl is required, while in the TaqMan 5 µl assays can be performed. This reduces the required amount of expensive Taq polymerase and fluorescently labeled probes to about ¼ compared to the LightCycler. In addition, cheap plastic ware microtiter plates replace costly optical glass capillaries. While the LightCycler is best suited for analysis of low sample numbers, the ABI prism 7900HT is an ideal instrumentation for large-scale association studies and high-throughput analysis.

While genotyping of larger cohorts with LightCycler was extremely laborious and prone to errors since pipeting and recording of results had to be done by hand, with the new established workflow a huge amount of samples could be genotyped most efficiently. With 3 LightCyclers an amount of 960 samples could be processed per day and the high-throughput workflow was capable to analyze nearly 40.000 samples in 24 hours. The established high-throughput workflow was used in various other large-scale association studies, which are not part of this work, where this level of throughput was actually reached. Only the number of thermal cyclers (6) limited capacity, the use of additional cyclers could increase throughput beyond that level.

Results obtained from genotyping of ABCA1 polymorphisms suggest, that the M771 is more prevalent in Italians and Hungarians compared to Germans. In Hungarians, a significantly decreased prevalence of V/M771 heterozygotes has been found in CAD patients (97.3%) compared to healthy controls (90.2%). Therefore, the introduced methionine at codon 771 might have a protective effect on development of coronary

artery disease in Hungarians. This fits well with the observation made in the M771 homozygous patient A. She had extremely high HDL levels, which should decrease risk to develop CAD.

In Italian centenarians, the K219 allele was found increased (48.7% carriers) compared to Italian donors (41.4% carriers). Therefore the K219 allele might represent a protective allele leading to longevity. It is noteworthy, that the R219K polymorphism in the Italian control group was in Hardy Weinberg disequilibrium. Polymorphisms should usually be in Hardy Weinberg equilibrium (HWE) assuming mainly that natural selection is not occurring and that the population is infinitely large. A marker that is not in HWE in case groups is a strong indication for natural selection and the importance of this marker. Since the control group is not in HWE, this might be an indication that either the assay produced false results or that the individuals do not display a representative cross-section of the population. The developed assay showed increased spreading of signals compared to other assays (Fig. 5.6) and a few falsely assigned results were detected during comparison of TaqMan and Luminex assay for this SNP (Tab. 5.17). Therefore, it seems possible, that a minor amount of samples was genotyped incorrectly. It is also conceivable, that this cohort did not contain enough individuals (N=150) or that the selected healthy blood donors are not a representative cross-section of the population. If the Hardy Weinberg disequilibrium is due to natural selection, sensitivity of the assay or constitution of the cohort remains unclear. However, the assumption that K219 has a protective effect in aging is in agreement with findings from other groups. European carriers of K219 (59% versus 50%) were found to have reduced severity of arteriosclerosis, a trend to increased HDL and fewer coronary events, which should clearly be life prolonging characteristics.[109]

6.3 Development of a bead-based multiplex assay

A new concept of bead-based nucleic acid testing with high multiplexing capability could be established in this thesis. Unlike LightCycler and TaqMan analysis, the Luminex method requires an initial PCR and purification step prior to allelic discrimination reaction. Yet its clear advantage is that a number of markers can be analyzed simultaneously, which could highly decrease the hands-on-time. In addition, analytical cost can be cut. The PCR reactions can be scaled down to 5 µl and several analyses can be performed in one reaction. Cheap standard (non-labeled) primers are required for allelic discrimination and only modified (biotinylated) dCTP is universally used for all discrimination reactions.

Due to handling of beads and centrifugation steps in this assay procedure, the method is not fully suited for a completely automated workflow, but since standard microtiter

plates are used, it could be integrated in a semi-automated workflow. This could further decrease the hands-on-time and increase reliability of test results.

It has been shown, that the newly developed multiplex assay is comparable to the firmly established TaqMan technology. Concordances of above 95.6% could be obtained within a 95% confidence interval (see Tab. 5.16). One Luminex assay (E1172D) produced a slightly increased amount of dropouts, another one (R219K) was more sensitive than the equivalent TaqMan assay. The two other assays are comparable. Two false assignments have been made by the Luminex method, which are due to false gating of obtained test results (see Fig. 5.10). By a more careful assignment, neglecting ambiguous results or further adjustment of gates, these errors could have been avoided. However, this experience could not be made prior to evaluation. Individual adjustment of gates for each assay should therefore be made in the future. The TaqMan technology was chosen for the evaluation of this novel assay system, since the newly established high-throughput workflow could readily provide analysis results. DNA sequencing and genotyping by sequence alignment without automated data processing would have been too laborious, prone to errors due to lack of automation and very costly. Therefore only discrepant results obtained by Luminex and TaqMan technology were analyzed by means of DNA sequencing. All methods are subject to failure due to quality of DNA and unexpected sequence variation in DNA that could influence correct genotyping. During the course of this work, occasionally false results have been obtained when genotyping V/M771 heterozygotes by DNA sequencing. The reason for this was that another SNP coupled with the M771 variant was located at a primer binding site, which caused inefficient binding of the primer and insufficient amplification of one allelic variant in PCR (data not shown). This illustrates, that all methods have their limitations and therefore, it is problematic to find a “gold standard“ in SNP analysis for the evaluation of a novel method. Thus it is imperative to apply a third method, when obtaining discrepant results.

Altogether, it can be concluded, that the bead-based multiplexing assay in the ASPE and zip code format provided test results with a sensitivity and accuracy comparable to the well-established TaqMan technology. While the LightCycler has its clear advantage in the fast analysis of a low number of samples and the TaqMan (7900HT) is ideally suited for high-throughput genotyping, this method is appropriate for a medium amount of samples (96-192), of which more than two markers have to be determined.

6.4 Final remark

HDL cholesterol levels and disease related to HDL might genetically be determined by a multitude of markers and it will be difficult and laborious to determine all of these parameters due to weak genotype-phenotype correlation. With a number of genes

playing an important role in reverse cholesterol transport and additional interacting proteins, which all harbor numerous sequence variations, there is still a lot of research to be done.

As RISCH stated: “The gene mutations studied by Mendel, and those more recently discovered by positional cloning, are those with large effect and strong genotype-phenotype correlation. They are effectively the ‘low-hanging fruit’ that are easy to harvest. Now, however, we are left with the great majority of the fruit at the top of the tree, with no obvious way to reach it.” [110]

This work did not intend to harvest a fruit from the top of this tree, but to provide pioneer work in identification of novel sequence variations in the ABCA1 gene and the development of assays for the analysis of SNPs in this gene.

To facilitate the analysis of novel markers for large-scale association studies, a high-throughput platform for the fast and efficient analysis of SNPs could be established. With the novel Luminex multiplexing method, an ideal platform for the efficient analysis of future multifactorial SNP profiles has been demonstrated.

7 Appendix

The appendix contains useful tables, figures and sequences, which have been prepared during the course of this work. Most of these figures and tables and additional material have been made publicly available in a web-page archive. The URL is: www.abca1-mutants.all.at

Tab. 7.1

List of known sequence variations in ABCA1 coding region (page 78)

This list contains all sequence variations in the coding region of ABCA1, reported in the literature so far. The numbered variations are also illustrated in Fig. 7.1.

Fig. 7.1

Predicted structure of the ABCA1 protein product and sequence variations therein (page 82)

Illustration of location of sequence variations in the ABCA1 protein product. Numberings refer to Tab. 7.1.

Tab. 7.2

Sequence variations in non-coding region of ABCA1 (page 83)

This list contains all sequence variations in the non-coding region of ABCA1 (promoter, exon 1 and 2), reported in the literature so far. The numbered variations are also illustrated in Fig. 7.2.

Fig. 7.2

Promoter region and non-coding sequence upstream of start codon (page 85)

Illustration of location of sequence variations in the promoter and the non-coding regions of exon 1 and 2 in the ABCA1 protein product. Numberings refer to Tab. 7.2.

Tab. 7.3

Tabular summary of patients with HDL-deficiency syndrome (page 87)

Tab. 7.4

Primers for ABCA1 sequencing and fragment analysis (page 89)

Complete set of primers for sequencing of promoter and all coding region of ABCA1.

No	Author, original designation	Ref	Type	Exon/ Intron	Amino acid(s)	Aa Pos. NM005502	Nucleo-tide(s)	mRNA NM005502	DNA AF275948	associated with
1	Altilia IVS2+5 G/C	[1]	PM	I 2	–	–	**G→C**	–	25,994	TD, SM, CAD
2	Ho Hong (Alabama)	[2]	PM	E 4	P→L	85	C**C**G→C**T**G	567	45,105	FHA, CAD
3	–	–	SNP	E 6	L	158	CT**G**→CT**A**	787	67,781	
4	Wang R/K159 Brousseau G596A Clee R219K	[3] [4] [5]	SNP	E 7	R→K	219	A**G**G→A**A**G	969	70,943	CAD
5	Wang R170C	[3]	PM	E 7	R→C	230	C**G**T→**T**GT	1,001	70,975	FHA
6	Nishida A255T	[6]	PM	E 8	A→T	255	**G**CT→**A**CT	1,084	84,004	TD
7	Brousseau TD17	[7]	Del	E 9	LFSMRSWSD→HEKLEX	272-80	Δ(TGTTCAGC)	1,128-35	89,011-18	TD, CAD
8	Altilia R282X	[1]	PM	E 9	R→X	282	C**G**A→**T**GA	1,157	89,040	TD, SM, CAD
9	Wang 676C/T	[3]	SNP	E 9	P	312	CC**C**→CC**T**	1,249	89,132	
10	Wang 888G/A	[3]	SNP	E 9	G	316	GG**G**→GG**A**	1,261	89,144	
11	Bodzioch TD4 Brousseau TD4 Clee V399A	[8] [7] [5]	SNP	E 11	V→A	399	G**T**G→G**C**G	1,509	92,434	(TD, SM)
12	Guo Case1(1)	[10]	Del/Ins	I 12-14	ΔE13,14	-Δ1255nt,Ins		1,823-2,205	97,551-98,806	TD SM, CAD
13	Rust Chile TD	[9]	Del/Ins	E 13	Δ(DERKFW),Ins(EYSGVTSAHCNLCLLSSS DSRASASQVAGITAPATTPG)	528-33	Δ14nt, Ins110nt	1,897-920	97,776-89	TD
14	Lawn TD2 Bertolini R527W	[11] [12]	PM	E 14	R→W	587	CGG→TGG	2,072	98,471	TD, CAD
15	Bodzioch TD5	[8]	PM	E 14	W→S	590	T**G**G→T**C**G	2,082	98,481	TD, SM
16	Probst Pat. C	[13]	PM	E 14	W→L	590	T**G**G→T**T**G	2,082	98,481	FHA
17	Brooks-Wilson TD2 Lawn TD1 Clee Q597R	[14] [11] [15]	PM	E 14	Q→R	597	C**A**G→C**G**G	2,103	98,502	TD
18	Bodzioch TD1 Rust German-TD	[8] [9]	Del	E 14	ITGTEKKTGVYMQQMPYPCYVDDIFLRV...→ LRAPRRKLVSICNRCPIPVTILMTSFCGX	608-635	ΔG	2,137	98,536	TD, CAD

Tab. 7.1
List of known sequence variations in ABCA1 coding region.

No	Author, original designation	Ref	Type	Exon/ Intron	Amino acid(s)	Aa Pos. NM005502	Nucleo-tide(s)	mRNA NM005502	DNA AF275948	associated with
19	Rust Swiss fam.	[9]	PM	E 14	Y→X	627	TA**C**→TA**G**	2,194	98,593	TD
20	Wang 1980C/A	[3]	SNP	E 15	I	680	AT**C**→AT**A**	2,353	100,538	
21	Brooks-W. FHA1 Clee Del L693	[14] [15]	Del	E 15	ΔΛ	693	Δ(CTT)	2,390-2	100,575-77	FHA
22	Clee V771 M Probst Pat. A.	[5] [13]	SNP	E 16	V→M	771	**G**TG→**A**TG	2,624	102,555	high HDL
23	Clee T774P	[5]	SNP	E 16	T→P	774	**A**CA→**C**CA	2,633	102,564	
24	Clee K776N	[5]	SNP	E 16	K→N	776	AA**G**→AA**C**	2,641	102,572	
25	Guo Case1(2)	[10]	Del/Ins	I 16-31	ΔE17-31	-	Δ19,6kb	2,651-4,777	103,621-23,566	TD, SM, CAD
26	Wang V/I765 Clee V825I	[3] [5]	SNP	E 17	V→I	825	**G**TC→**A**TC	2,786	103,777	
27	Probst Pat. B	[13]	PM	E 17	W→R	840	**T**GG→**A**GG	2,831	103,822	TD
28	Wang I/M823 Brousseau A2589G Utech 2	[3] [4] [16]	SNP	E 18	I→M	883	AT**A**→AT**G**	2,962	105,057	increased HDL
29	Marcil fam4 Clee R909X	[17] [15]	PM	E 19	R→X	909	**C**GA→**T**GA	3,038	106,930	SM FHA
30	Lapicka C2665del	[18]	Del	E 19	RDGMK...→EMGX	909-12	ΔG	3,038	106,931	TD, SM
31	Clee T929I	[5]	SNP	E 19	T→I	929	A**C**C→A**T**C	3,099	106,991	no CAD
32	Guo Case2	[10]	PM	E 19	N→H	935	**A**AT→**C**AT	3,116	107,008	TD, SM
33	Bodzioch TD3 Guo Case3 Utech 1	[8] [10] [16]	PM	E 19	N→S	935	A**A**T→A**G**T	3,117	107,009	TD, SM
34	Bodzioch TD4/5	[8]	PM	E 19	A→V	937	G**C**G→G**T**G	3,123	107,015	TD, SM
35	Wang 2820G/A	[3]	SNP	E 20	L	960	CT**G**→CT**A**	3,193	108,074	
36	Wang A986D	[3]	PM	E 21	A→D	1,046	G**C**C→G**A**C	3,450	109,839	TD
37	Probst Pat. D	[13]	PM	E 22	W→R	1,068	**C**GC→**T**GC	3,515	109,904	TD
38	Marcil fam5 Clee M1091T	[17] [15]	PM	E 23	M→T	1,091	A**T**G→A**C**G	3,585	110,676	CAD-FHA

Tab. 7.1
List of known sequence variations in ABCA1 coding region (contd.).

No	Author, original designation	Ref	Type	Exon/ Intron	Amino acid(s)	Aa Pos. NM005502	Nucleo-tide(s)	mRNA NM005502	DNA AF275948	associated with
39	Rust Swiss family	[9]	Ins	E 23	D...→...X	1,095-114	Ins(G)	?	?	TD
40	Ho Hong D1099Y	[19]	PM	E 23	D→Y	1099	GAC→TAC	3,608	110,699	TD
41	Remaley Δ3283-4	[20]	Del	E 23	SSLFLKNQLGTGYYLTLVKKDVESSLSSCRN...→FVSEEPAGNRLLPDLGQERCGILPQFLQKQX	1,114	Δ(TC)	3,653-4	110,744-5	TD
42	Brousseau G3456C Clee E1172D	[4] [5]	SNP	E 24	E→D	1,172	GAG→GAC	3,829	112,177	
43	Brooks-Wilson Clee IVS25+ 1G→C	[14] [15]	PM	I 25	-	-	GT→CT (splice site)	-	113,386	TD, CAD
44	Huang FHA	[21]	Del	E 27	RPFTEDDAADPNDSDI→LSLKMMLLIQMILTX	1,283-98	Δ(CGCC)	4,160-3	115,356-9	FHA, CAD
45	Brousseau TD18 Huang TD1	[7] [21]	PM	E 27	D→N	1,289	GAT→AAT	4,178	115,374	TD, SM
46	Wang 4221G/A	[3]	SNP	E 31	T	1,427	ACG→ACA	4,594	123,100	
47	Brooks-Wilson TD1 Clee C1477R	[14] [15]	PM	E 31	C→R	1,477	TGT→CGT	4,742	123,248	TD, CAD
48	Lapicka C4457T	[18]	PM	E 32	S→L	1,506	TCG→TTG	4,830	124,856	TD, SM
49	Wang 4570insA	[3]	Ins	E 34	ALPPSQEVN...→STSSESRSX	1,544	Ins(A)	4,943	127,400	TD, CAD
50	Wang R/K1527 Clee R1587K	[3] [5]	SNP	E 35	R→K	1,587	AGA→AAA	5,073	129,004	
51	Nishida N1611D	[6]	PM	E 36	N→D	1,611	AAT→GAT	5,099	129,551	TD
52	Ishii R1708W	[22]	PM	E 37	R→W	1,680	CGG→TGG	5,351	131,033	TD
53	Lawn TD3(2)	[11]	Ins	E 38	C→?	1,708	Ins (138 nt)	5,435	133,114	CAD, neuropathy
54	Clee S1731C	[5]	SNP	E 38	S→C	1,731	TCC→TGC	5,505	133,184	
55	Bodzioch TD2	[8]	Del	I 39	ΔE40-50	1,795-2,262	?	5,696	?	TD, SM
56	Brousseau TD16	[7]	PM	E 40	N→H	1,800	AAT→CAT	5,711	134,541	TD, CAD neuropathy
57	Nishida R1851X	[6]	PM	E 41	R→X	1,851	CGA→TGA	5,864	135,780	TD

Tab. 7.1
List of known sequence variations in ABCA1 coding region (contd.).

Tab. 7.1
List of known sequence variations in ABCA1 coding region (contd.).

No	Author, orignial designation	Ref	Type	Exon/ Intron	Amino acid(s)	Aa Pos. NM005502	Nucleo-tide(s)	mRNA NM005502	DNA AF275948	associated with
58	Marcil fam3 Clee Del E,D 1893-4	[17] [15]	Del	E 42	ΔED	1,893-4	Δ(GAA GAT)	5,990-96	136,170-6	FHA
59	Lawn TD3(1)	[11]	Ins	E 42	G…→?	1,905	Ins(14 nt)	?	?	CAD, neuropathy
60	Ho Hong F2009S	[19]	PM	E 45	F→S	2,009	T**T**C→T**C**C	6,339	140,570	
61	–	–	SNP	E 46	G	-2,061	GG**C**→GG**T**	6,496	141,095	FHA
62	Huang TD2	[21]	PM	E 47	R→W	2,081	**C**GG→**T**GG	6,554	142,096	TD, SM
63	Marcil fam2 Clee 2144X	[17] [15]	PM	E 49	R→X	2,144	**C**GA →**T**GA	6,743	143,425	CAD, SM FHA
64	Clee Del C6825	[15]	Del	E 49	…I→…X	…-2,145	?	?	?	no CAD
65	Clee P2150L Probst healthy OCT	[15] [13]	PM	E 49	P→L	2,150	C**C**G→C**T**G	6,762	143,444	CAD
66	Probst Pat. E	[13]	PM	E 49	F→S	2,163	T**T**T→T**C**T	6,801	143,483	FHA
67	Brousseau TD19	[7]	Ins	E 49	SQSKKRLHIEDYSVSQTTLD…→ SFPEQKATPHRRLLCFSDNTX	2,195-214	Ins(CATT)	6,896	143,578	TD, SM
68	Clee CTC6952-4TT	[15]	Del/Ins	E 49	…I→…X	…-2,203	?	6,920-2	?	no CAD
69	Probst Pat. E	[13]	PM	E 50	V→I	2,244	**G**TT→**A**TT	7,043	144,665	

silent SNP (not affecting amino acid sequence)

SNP (amino acid exchange, no association with phenotype)

point mutation (single base variation resulting in different phenotype)

insertion / deletion

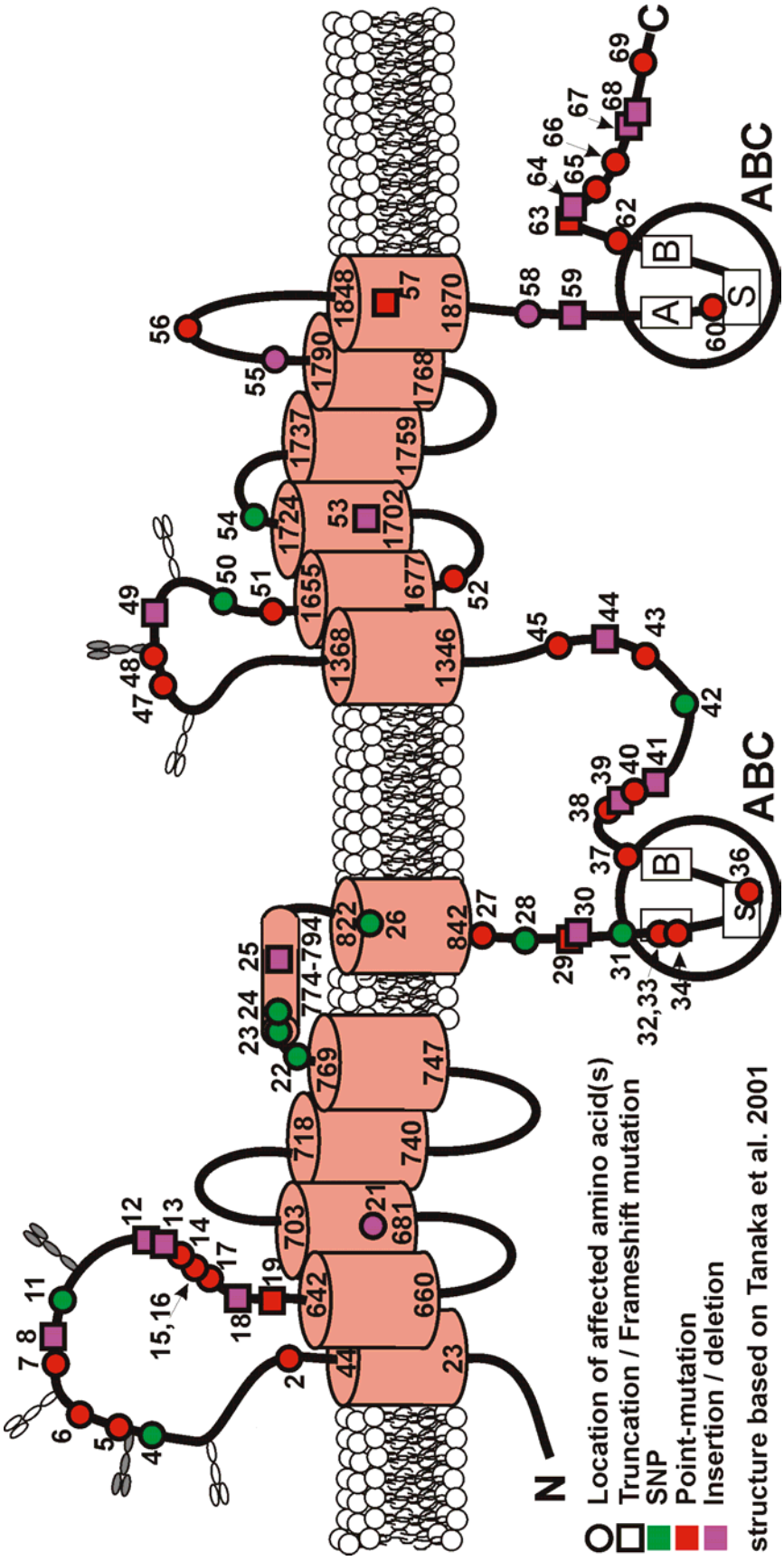

Fig. 7.1
Predicted structure of the ABCA1 protein product and sequence variations therein.

No	Variation	Ref	Type	Location	Nucleotide(s)	Pos.mRNA NM005502	Pos.DNA AF275948	associated with
N1	Probst VNTR-ZNF	[111]	VNTR	Promoter	Δ(GTTT)/Δ(GTTTTGTTT)	-	688-96	-
N2	Lutucuta –477C/T Kyriakou –565C/T	[112] [113]	SNP	Promoter	C→T	-	890	- CAD
N3	Lutucuta –419A/C	[112]	SNP	Promoter	A→C	-	948	-
N4	Probst G1047C	[111]	SNP	Promoter	G→C	-	1,047	-
N5	Probst C1152T	[111]	SNP	Promoter	C→T	-	1,152	-
N6	Pullinger G/C-191 Zwarts G-191C Probst C1176G	[89] [90] [111]	SNP	Promoter	GGGC→GGGG	-	1,176	CAD - -
N7	Probst C1214A	[111]	SNP	Promoter	C→A	-	1,214	-
N8	Probst VNTR-ZNF	[111]	VNTR	Promoter	Δ(ACCCC)	-	1,222-6	-
N9	Zwarts C-17G Probst C1355G	[90] [111]	SNP	Promoter	C→G	-	1,355	dec. CAD -
N10	Probst C1440T	[111]	SNP	Promoter	C→T	-	1,440	-
N11	Zwarts C117G Probst C1487G	[90] [111]	SNP	Exon 1	C→G	34	1,487	CAD -
N12	Zwarts InsCCCT-1163	[90]	Ins	Intron 1	Ins(CCCT)	-	24,668	-
N13	Zwarts A-1095G	[90]	SNP	Intron 1	A→G	-	24,736	-
N14	Zwarts G-1027A	[90]	SNP	Intron 1	G→A	-	24,805	-
N15	Zwarts G-720A	[90]	SNP	Intron 1	G→A	-	25,111	-

Tab. 7.2
Sequence variations in non-coding region of ABCA1.

No	Variation	Ref	Type	Location	Nucleotide(s)	Pos.mRNA NM005502	Pos.DNA AF275948	associated with
N16	Zwarts A-461C	[90]	SNP	Intron	C→A	-	25,370	-
N17	Zwarts A-362G	[90]	SNP	Intron	G→A	-	25,469	-
N18	Pullinger Ins/del319 Zwarts InsG319	[89] [90]	Del	Exon 2	GGG→GG	237	25,846	-
N19	Pullinger C/G378 Zwarts G378C	[89] [90]	SNP	Exon 2	C→G	296	25,906	-

no association found or known

association shown

Tab. 7.2
Sequence variations in non-coding region of ABCA1 (contd.).

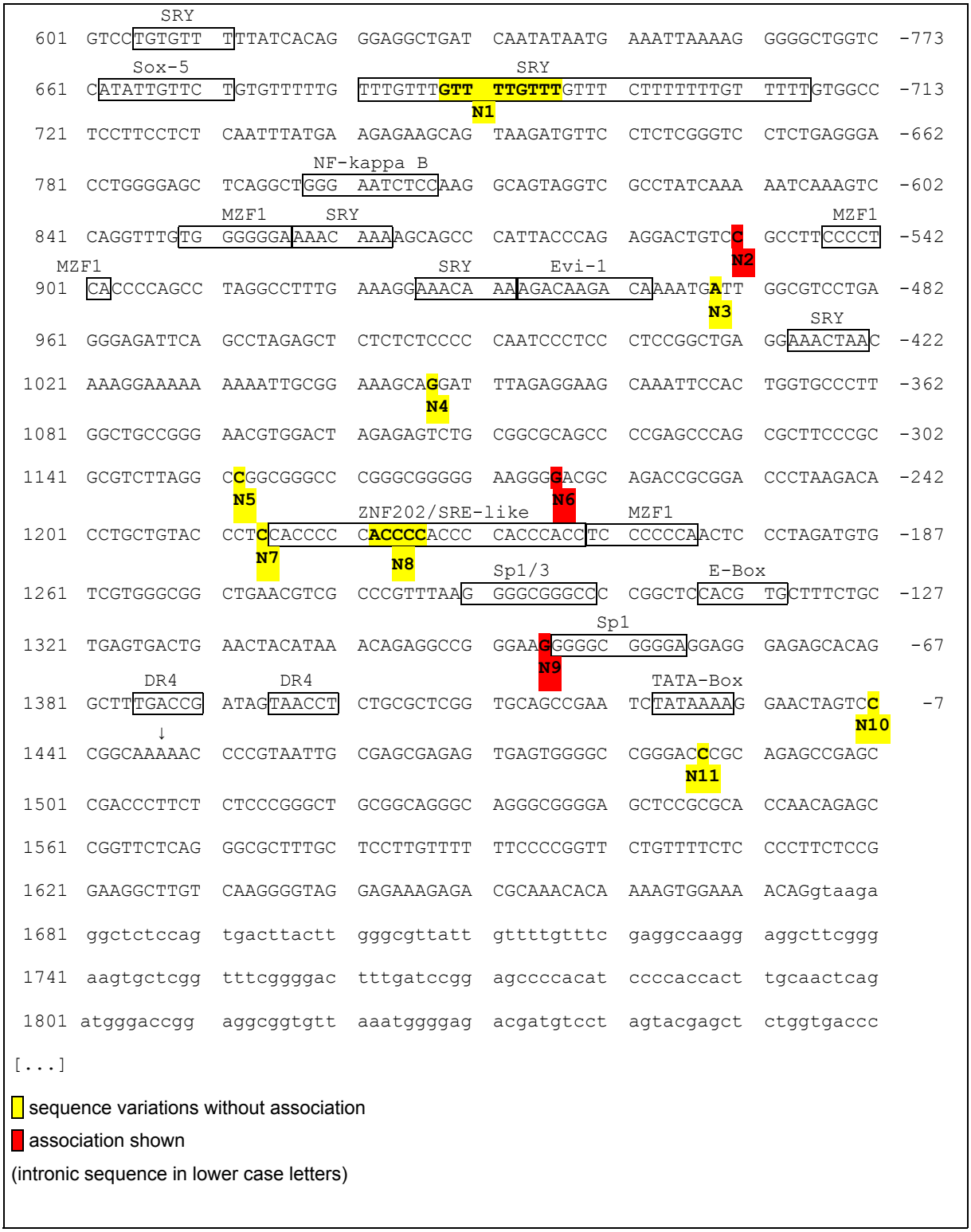

Fig. 7.2
Promoter region and non-coding sequence upstream of start codon.

```
[...]

24301  ggcatggaga  tctcatttgg  actcacagat  ttctagtcta  gcgcttggtt  ttgtatccat

24361  acctcgctac  tgcattctta  gttccttctg  ctccttgttc  ctcatgccca  gtgtcccacc

24421  ctacccttgc  ccctactcct  ctagaggcca  cagtgattca  ctgagccatt  tcataagcac

24481  agctaggaga  gttcatggct  accaagtgcc  agcagggccg  aattttcacc  tgtgtgtcct

24541  cccttccatt  tttcatcttc  tgccccctcc  ccagctttaa  ctttaatata  actacttggg

24601  actattccag  cattaaataa  gggtaactgc  tggatgggtg  gctgggatac  acagaatgta

24661  gtatccctTg  ttcacgagaa  gaccttcttg  ccctagcatg  gcaaacagtc  ctccaaggag
                N12
24721  gcacctgtga  cacccAgcgg  agtaggggggg  cggtgtgttc  aggtgcaggt  ggaacaaggc
                      N13
24781  cagaaagtgtg  catatgtgct  gaccGtggga  gcttgtttgt  cggtttcaca  gttgatgccc
                             N14
24841  tgagcctgcc  atagcagact  tgtttctcca  tgggatgctg  ttttctttcc  agagacacag

24901  cgctagggtt  gtcctcatta  cctgagagcc  aggtgtcggt  agcattttct  tggtgtttac

24961  tcacactcat  ctaaggcacg  ttgtggtttt  ccagattagg  aaactgcttt  attgatggtg

25021  ctttttttttt  ttttttgag  acagagtctc  gctctgtcgc  catgctggag  tgtagtggca

25081  caatcttggc  tcactgcacc  tccgcctgcc  gggttcagcg  attctcctgc  ctcagcctcc
                                      N15
25141  caagtagctg  ggactacagg  tgcctgccac  catgcccagc  taatttttgt  atttttagta

25201  gagacggggt  ttcaccgtat  tggctaggat  ggtctcgatt  tcttgacctc  gtgatccgcc

25261  tgcctcggcc  tcccaaagtg  ctgggattat  aggcttgagc  caccacgcct  ggccgatggt

25321  gctttttatc  atttgaagga  ctcagttgta  taacccactg  aaaattagtc  tgtaaggaag
                                                          N16
25381  ttcagggaat  agtataagtc  actccaggct  tgaggcaaaa  tttacaaatg  ctgctgactt

25441  tgtatgtaag  gggaggcatt  ttcttagaga  agagaggtag  gtctctggga  ttccagtatg
                                      N17
25501  ccatttccat  cctcagtgtt  tttggccacc  tgagagaggt  ctattttcag  aaatgcattc

25561  ttcattccca  gatgataaca  tctatagaac  taaaatgatt  aggaccataa  cacgtagctc

25621  ctagcctgct  gtcggaacac  ctcccgagtc  cctctttgtg  ggtgaaccca  gaggctggga

25681  gctggtgact  catgatccat  tgagaagcag  tcatgatgca  gagctgtgtg  ttggaggtct

25741  cagctgagag  ggctggatta  gcagtcctca  ttggtgtatg  gctttgcagc  aataactgat

25801  ggctgtttcc  cctcctgctt  tatctttcag  TTAATGACCA  GCCACGGGCG  TCCCTGCTGT
                                                    N18
25861  GAGCTCTGGC  CGCTGCCTTC  CAGGGCTCCC  GAGCCACACG  CTGGGCGTGC  TGGCTGAGGG
         START                                                 N19
25921  AACATG...
```

sequence variations without association

association shown

(intronic sequence in lower case letters)

Fig. 7.2
Promoter region and non-coding sequence upstream of start codon (contd.).

Case	Kindred	Age [year]	Sex	Mutation	CAD	SM	TC	TG	VLDL-C	LDL-C	HDL-C	ApoA-I	ApoA-II	Apo B	References
1	1	66 [1999]	F	#18	+	–	142	376	–	108	1	<20	<4.6	–	[17, 18, 114]
2		63 [1999]	M	#18	+	–	90	304	–	27	2	<20	<4.6	–	[17, 18, 114]
3		60+ [1999]	F	#18	+	–	153	411	–	84	1	<20	<4.6	–	[17, 18, 114]
4	2	52 [1999]	M	#55	–	+	43	386	–	14	1	<20	<4.6	–	[17]
5		49 [1999]	M	#55	–	+	70	481	–	22	2	<20	<4.6	–	[17]
6	3	69 [1999]	F	#33	–	+	128	362	–	54	2	<20	<4.6	–	[17]
7		66 [1999]	M	#33	–	+	58	245	–	8	1	<20	<4.6	–	[17]
8	4	39+[1999]	M	#11 / #34	–	+	201	193	–	183	3	<20	<4.6	–	[17]
9		37 [1999]	M	#11 / #34	+	?	88	570	–	27	1	<20	<4.6	–	[17]
10	5	52 [1999]	M	#15 / #34	+	?	132	169	–	90	8	27	12.1	–	[17]
11	6	43[1999]	M	#42 / #47	+	–	–	76	–	–	<4	–	–	–	[19, 115]
12	7	42 [1977]	M	#33	–	+	45	300	–	–	0	–	–	–	[114, 116-120]
13		46 [1977]	F	#33	–	–	75	320	–	–	<5	–	–	–	[114, 116-120]
14	8	?	?	#13	–	–	–	–	–	–	–	–	–	–	[18]
15	9	?	?	#19 / #39	?	?	–	–	–	–	–	–	–	–	[18, 115]
16	10	56 [1999]	M	#17	–	+	72	297	–	–	6	1	2	110	[19, 27, 119,
17	11	48 [1999]	F	#14	+	–	102	120	–	–	2	6	5	–	[27, 118, 121]
18	12	18 [1999]	M	#53 / #59	–	–	–	–	–	16	5	–	–	–	[27]
19	13	24[1994] 29[1999]	M	#41	–	–	53 35	284 352	37 –	16 28	0 8	– 3	– 2	– –	[18, 91, 115, 119]
20		25[1994]	F	#41	–	–	63	351	33	30	0	–	–	–	[18, 91, 115,
21	14	52 [1994]	F	#56	+	–	115	185	22	8.6	3	–	–	–	[115, 119]
22	15	51 [2000]	M	#7 / ?	+	–	36	333	–	–	1	–	–	–	[115]
23	16	28 [1994]	F	#45	–	–	50	175	34	15	8	–	–	–	[115, 119]
24		26 [1994]	F	#45	–	–	39	132	67	–	8	–	–	–	[115, 119]
25	17	52 [2000]	M	#38 / ?	–	+	72	307	–	–	3	–	–	–	[115]
26	18	38 [2000]	M	#49 / #36	+	–	107	–	–	–	3	<4.5	–	–	[95]
27		46 [2000]	M	#49 / #36	?	?	51	–	–	–	2	<5.4	–	–	[95]
28		40 [2000]	F	#49 / #36	?	?	121	–	–	–	3	<5.4		–	[95]
29	19	57 [2002]	M	#12 + #25	+	+	22	88	–	–	4	3.2	3.5	–	[123]
30	20	69 [2002]	M	#32	–	+	34	187	–	–	0.8	<5	<2	–	[123]
31	21	20 [2002]	M	#33	–	+	61	114	–	–	0	0	0.3	–	[123]
32	22	69 [2002]	M	#40 / #60	–	–	184	130	–	154	4	8	–	168	[124]
33	23	60 [2002]	M	#2 / ?	+	–	141	224	–	91	5	23	114	–	[125]
34	24	48 [2002]	M	#52	+	–	96	170	–	56	5	7.0	8.8	90	[126]
35	25	66 [2003]	F	#1 / #8	+	–	109	191	–	–	3	<10	5	93	[127]
36	26	50 [2003]	F	#27 / #33	–	–	110	452	57	48	4	<4	–	110	[111]
37	27	61 [2003]	M	#16 / ?	+	–	–	–	–	–	4	<4	–	–	[111]
38	28	32 [2003]	M	#37 / 41	?	?	49	316	15	24	2	5	–	56	[111]

Tab. 7.3
Tabular summary of patients with HDL-deficiency syndrome.

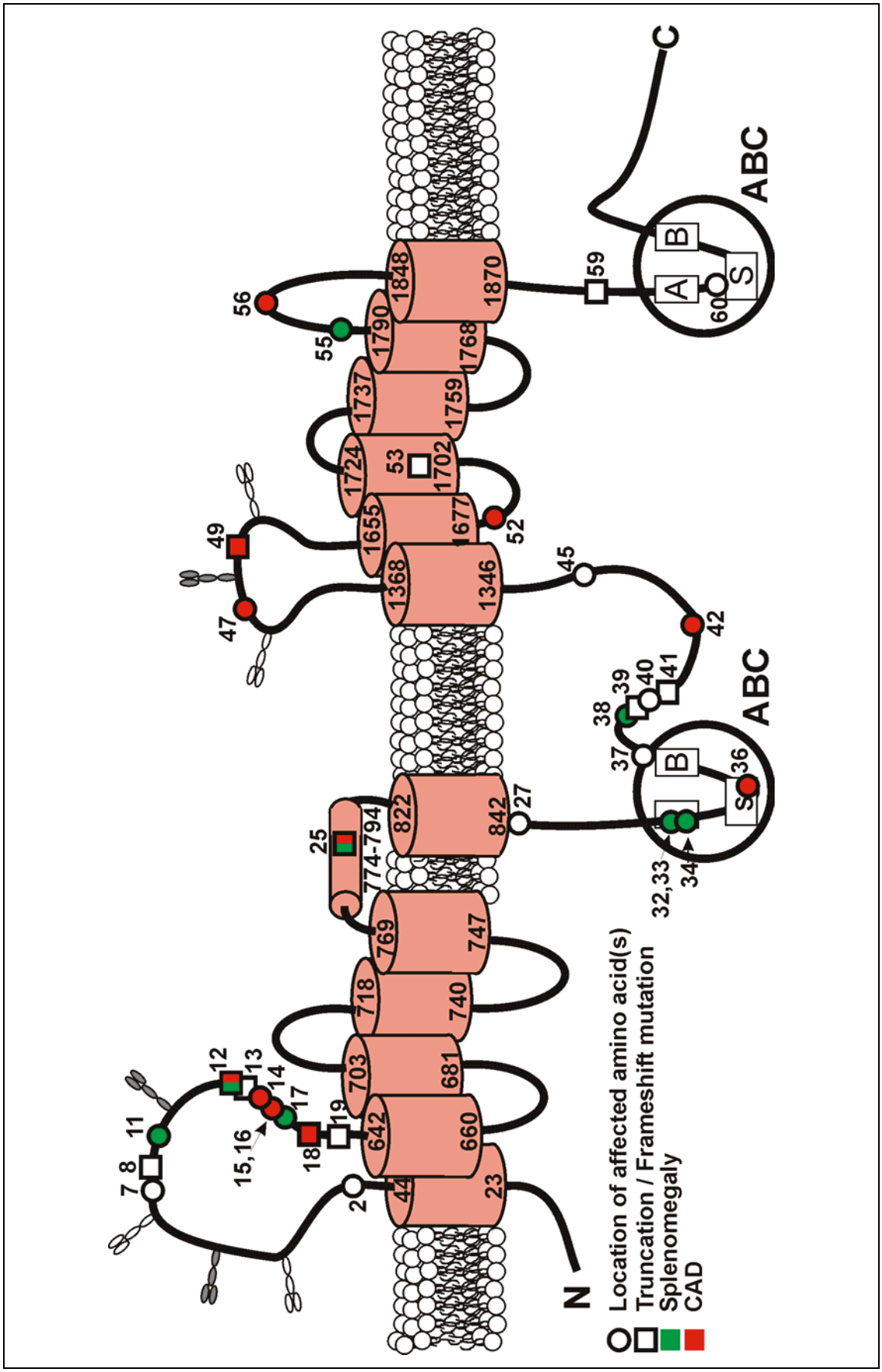

Fig. 7.3
Location of mutations in ABCA1 found in patients with HDL-deficiency syndrome (Tab. 7.3) and their connection to splenomegaly and CAD.

Exon	Primer name	Primer sequence	Exon size (bp)	Size of amplicon (bp)
prom1	ABCA1-pro1-f	GGA GGT CTG GAG TGG CTA CAT A		554
	ABCA1-pro1-r	CCA GTG GAA TTT GCT TCC TCT A		
prom2	ABCA1-pro2-f	AGC CCA TTA CCC AGA GGA CTG T		466
	ABCA1-pro2-r	TTC AGT CAC TCA GCA GAA AGC A		
prom3	ABCA1-pro3-f	CCC TAA GAC ACC TGC TGT ACC C		380
	ABCA1-pro3-r	CTG AGA ACC GGC TCT GTT GGT		
prom4	ABCA1-pro4-f	GGA GGT CTG GAG TGG CTA CAT		246
	ABCA1-pro4-r	AGA GGA CCC GAG AGG AAC AT		
1	ABCA1-ex1-f	AGC ACA GGC TTT GAC CGA TAG T	221	395
	ABCA1-ex1-r	GGA TCA AAG TCC CCG AAA CC		
2	ABCA1-ex2-f	TCC TCA TTG GTG TAT GGC TTT G	158	358
	ABCA1-ex2-r	AAG AGC CAG ATT CCA TCA ATC C		
3	ABCA1-ex3-f	CCA CGA TGA ATC CTG AAG AAT G	94	540
	ABCA1-ex3-r	CTC CTG AGT TCA TGC CTT ATC A		
4	ABCA1-ex4-f	ATC ACT TCT CCA ACG CTG GTA T	142	519
	ABCA1-ex4-r	TGC AGA CTC TAT CAC ACA AGC A		
5	ABCA1-ex5-f	AGG CAG TTG GCC TAG CTA AAG	119	236
	ABCA1-ex5-r	CTA CTC TCT TTC CCT GGT GCA G		
6	ABCA1-ex6-f	GCC TAT CAT GGT GAA ACC CTG T	122	297
	ABCA1-ex6-r	TCT TTC CAG TAG CTG CAC AAC G		
7	ABCA1-ex7-f	AAA GAC TTC AAG GAC CCA GCT T	177	321
	ABCA1-ex7-r	CCT CAC ATT CCG AAA GCA TTA		
8	ABCA1-ex8-f	TTT GGT TAG GAG TCG GTT TCT TG	93	456
	ABCA1-ex8-r	GGG CTG GTC TGT TAG CCA CTA		
9	ABCA1-ex9-f	TCT TTC TCT TCT CAT CCC CAA C	241	417
	ABCA1-ex9-r	GCT GCT ACA GAG GGA GGA GAT		
10	ABCA1-ex10-f	TCC AAA GCC CTT GTA AGT TTC T	140	305
	ABCA1-ex10-r	TAC TTA GCG CAC ACC TCT GAA G		
11	ABCA1-ex11-f	CTC ATT GTC TGT GCT TCT CCT C	117	240
	ABCA1-ex11-r	GTG ACC AGA AAC TCA CCT CTC C		
12	ABCA1-ex12-f	TGC TCC TCA AGA TTT AGT TGG TG	198	306
	ABCA1-ex12-r	TCA GAG AAA GAA GCC GTT AAG TC		
13	ABCA1-ex13-f	GAT GAG CAA TCG TGT AGT CAG C	206	487
	ABCA1-ex13-r	ACC TTC CAG CAA GTC ATG TAC C		
14	ABCA1-ex14-f	GGG CAA TAA GAG TGA AAC TCC A	177	438
	ABCA1-ex14-r	TTT CCA GAT CAT TCA CAC ATG C		
15	ABCA1-ex15-f	AGA GTC TAG AGG GCC TGT CCT T	223	593
	ABCA1-ex15-r	GAA CCC TGC ACT CTC ATC CTA C		
16	ABCA1-ex16-f	TAC AAG TGA GTG CTT GGG ATT G	222	391
	ABCA1-ex16-r	CCC ATT GGA AAA GAC AAT CAT C		
17	ABCA1-ex17-f	TTC TGC ACC TTA TGA TTG ATC C	205	392
	ABCA1-ex17-r	AGC ACA AAG AAA GGA CAT CAG C		
18	ABCA1-ex18-f	CAG AAT ACT GGG ATG ATG CTG A	114	227
	ABCA1-ex18-r	AAC AGT TAG CAG AGG CAG CAG		
19	ABCA1-ex19-f	TGT CCT TAC ACT CCA CTC CTC A	172	292
	ABCA1-ex19-r	CTC TAC TGC AGA ACC CTC CTG T		
20	ABCA1-ex20-f	AAG CAG ATG TGA GAA GCA CCT G	132	868
	ABCA1-ex20-r	TAA GTC CCA CTC CTC CCA TGA T		
21	ABCA1-ex21-f	CGA TCT GTC ACC TTT CAC TTT G	143	490
	ABCA1-ex21-r	GCC AGG GAC AAG TTT CTG TTA C		
22	ABCA1-ex22-f	CTC TGC CTT CAC TCA CTG TCT G	138	283
	ABCA1-ex22-r	TAG CCA TGA GAT ACA GCC ACA C		
23	ABCA1-ex23-f	TGT GAC AAA TCA GAA CTG AGA GG	221	366
	ABCA1-ex23-r	GGT GGA GAT GGA GAA ATC ATT C		

Tab. 7.4
Primers for ABCA1 sequencing and fragment analysis, partly published in [128].

Exon	Primer name	Primer sequence	Exon size (bp)	Size of amplicon (bp)
24	ABCA1-ex24-f	CAC ACA ACA GAG CTT CTT GGA A	73	257
	ABCA1-ex24-r	TCT GCA CCT CTC CTC CTC TG		
25	ABCA1-ex25-f	GGA ATG ACC TAA ACA CCT GGA A	204	402
	ABCA1-ex25-r	AGG CTA CTG GTC TGG CCT TAG		
26	ABCA1-ex26-f	GGC TTC TCT CCC AGG TAA CTC T	49	404
	ABCA1-ex26-r	GCA CAA GGA AAG AAT CCC ATA C		
27	ABCA1-ex27-f	TCA GTG CAC GAG TAT TGT TCC T	114	400
	ABCA1-ex27-r	TGG CTT TTA CCA TTT CCT CAC T		
28	ABCA1-ex28-f	TTC AGA CAC ATG CCT TCA AGA T	149	403
	ABCA1-ex28-r	GCA AGG AAT AGA ACT GGG ATT G		
29	ABCA1-ex29-f	CTC AGT CTT CAG GAG GAG GAA G	125	517
	ABCA1-ex29-r	CAT CTT TGG TCT GCT CGA ATC		
30	ABCA1-ex30-f	TTG GAG TGC TCT ACG TCA CC	99	402
	ABCA1-ex30-r	GTC ACT AAT GTG GCA TGC AGT T		
31	ABCA1-ex31-f	GTT TGT GGT TGT TAC GGA ATG A	190	390
	ABCA1-ex31-r	AAC AGA CCC TCC CAA CAT GAT A		
32	ABCA1-ex32-f	TCT GGG ACC TGT AGT CAG GTT T	95	412
	ABCA1-ex32-r	CCT TGA AGC TGA CAA CTG AAT G		
33	ABCA1-ex33-f	TGC ATC ATT AGG AAT AGG CTC A	33	401
	ABCA1-ex33-r	CCT GCA GGA GAT TTC TGA TTC T		
34	ABCA1-ex34-f	GCA GAA ACC ATG GGA GTT AAG A	106	380
	ABCA1-ex34-r	TCC GTT TAA CCT GCC AAC TAC T		
35	ABCA1-ex35-f	GTC CAC AGG TTC CAG ATT GAC T	75	304
	ABCA1-ex35-r	ACT ACA CCG TAC TGC CTC ACT G		
36	ABCA1-ex36-f	GTT GGT GGG TAT GCA CCT G	170	305
	ABCA1-ex36-r	TCT CCA TAA CCC TCT CCC TTG		
37	ABCA1-ex37-f	ACT GAT TGA GCC CTA ACT GCA T	178	408
	ABCA1-ex37-r	TGT GTT TGG TGT CAT GAA AGT G		
38	ABCA1-ex38-f	GCT TGA CTG TTG GAA AGA CAG A	116	402
	ABCA1-ex38-r	CAC CAA ATG CCT TAT CCA CTG		
39	ABCA1-ex39-f	ACA GTG GAT AAG GCA TTT GGT G	145	400
	ABCA1-ex39-r	AGC AAG AAA GAA AGG TGC TTT G		
40	ABCA1-ex40-f	TTC TCA CTT GCC TAG TGG ATT G	124	860
	ABCA1-ex40-r	ATC CTG TGC TTA GTC ACC TGC T		
41	ABCA1-ex41-f	CGA ATA CAC ATT GGC TCT GAA A	130	464
	ABCA1-ex41-r	TTG AGT TCA GTT CAA TGC AAC C		
42	ABCA1-ex42-f	GGA GAC TGT GGC AAG TAG GTT T	121	442
	ABCA1-ex42-r	TGT ATT CGA GAA TGG GAA TTC AT		
43	ABCA1-ex43-f	GCC TGT GTT CAA GAG GGT CTA T	63	565
	ABCA1-ex43-r	CGT TGC TTG ATT GGG TAG AGA		
44	ABCA1-ex44-f	GTT TGA GGT AGT TAC GTG TTA GGG	107	516
	ABCA1-ex44-r	AAC TGG ATT GTG GTG ATG GTT G		
45	ABCA1-ex45-f	GCA TGT GAA TGG TGC ATA TGA G	142	567
	ABCA1-ex45-r	GTC AGG ATG CCA AAG GAG ACA G		
46	ABCA1-ex46-f	CCT CAC CAG TTG TCA GGT TAT G	135	512
	ABCA1-ex46-r	AGC CTC AAT CAC GCT AAG AAA C		
47	ABCA1-ex47-f	GCT GTT TCA AAG ATG CTT CTG C	104	236
	ABCA1-ex47-r	AAA CAT CCC ACA GTG AGG AAC		
48	ABCA1-ex48-f	AGT CAT GGT GAT GTT CTC ATG G	93	309
	ABCA1-ex48-r	GGA CTT CAA AGC CCT CAT TCT T		
49	ABCA1-ex49-f	ACT TCA TGG GTC CAG GTG AG	244	528
	ABCA1-ex49-r	GGA ATC CAC ACC CTG AGA AGT A		
50	ABCA1-ex50-f	CTT CCC TCC TTC ACA CAC AGT T	141	500
	ABCA1-ex51-r	GAC ATA GGC TAC AAA GGC ACT G		

Tab. 7.4
Primers for ABCA1 sequencing and fragment analysis, partly published in [128] (contd.).

8 Abbreviations, acronyms and symbols

A ..... Adenine
ABC ..... ATP-binding cassette
APD ..... Avalanche photo diode
apo E ..... Apolipoprotein E
ARMS ..... Amplification refractory mutation system
ASO ..... Allele specific oligonucleotide
ASPE ..... Allele specific primer extension
ATP ..... Adenosine triphosphate
a.u. ..... Arbitrary units
BADGE ..... Beads array for detection of gene expression
bp ..... Base pairs (of DNA)
C ..... Cytosine
CAD ..... Coronary artery disease
cAMP ..... Cyclic adenosine monophosphate
Cdc42 ..... Cell division cycle 42 GTP binding protein
CE ..... Cholesteryl ester
CENT ..... Cohort of Italian centenarians
CETP ..... Cholesteryl ester transfer protein
CFTR ..... Cystic fibrosis transmembrane conductance regulator
contd. ..... Continued
G ..... Guanine
GTP ..... Guanosine-triphosphate
CVD ..... Cardiovascular disease
CYP ..... Cytochrome P450 enzyme
ddNTP ..... Dideoxynucleotide triphosphate
Del. ..... Deletion
dNTP ..... Deoxynucleotide triphosphate
DON_H ..... Cohort of healthy Hungarian blood donors
DON_I ..... Cohort of healthy Italian blood donors
DOL ..... Dye-labeled oligonucleotide ligation assay
DR4 ..... Direct repeat separated by four bases
ELISA ..... Enzyme linked immunosorbent assay
EMSA ..... Electromobility shift assay
FEN ..... Flap endonuclease
FI ..... Fluorescence intensity
Fig. ..... Figure

FLISA ..... Fluorescence-linked immunosorbent assays
FRET ..... Fluorescence resonance energy transfer
HCV ..... Hepatitis C-virus
HDL (-C)..... High density lipoprotein (- cholesterol)
HIV ..... Human immunodeficiency virus
HNF1α ..... Hepatic nuclear factor 1α
HL ..... Hepatic lipase
HLA ..... Human Leukocyte Antigen
HSV ..... Herpes simplex virus
HWE ..... Hardy-Weinberg equilibrium
IDL ..... Intermediate density lipoprotein
Ins. ..... Insertion
KRAB ..... Krüppel-associated box domain (of ZNF202)
LAT ..... Latex agglutination test
LCAT ..... Lecithin:cholesterol acyltransferase
LDL ..... Low density lipoprotein
LPL ..... Lipoprotein lipase
LXR ..... Liver X receptor
MALDI-TOF ..... Matrix assisted laser desorption ionisation – time of flight
MDR1 ..... Multi drug resistance gene 1
MGB ..... Minor groove binder
MRP1 ..... MDR-associated protein 1
MTHFR ..... Methylene Tetrahydrofolate Reductase
N ..... Number of individuals of specified cohort
NBD ..... Nucleotide binding domain
NFQ ..... Non-fluorogenic quencher
NSF ..... N-ethylmaleimide-sensitive fusion
OLA ..... Oligonucleotide ligation assay
(P) ..... Phosphate (phosphorylation of 3’ hydroxyl group)
PC ..... Phosphatidylcholine
PCR ..... Polymerase chain reaction
PLTP ..... Phospholipid transfer protein
PMT ..... Photo multiplier tube
PPi ..... Pyrophosphate
RCA ..... Rolling-circle amplification
Ref. ..... Reference(s)
RFLP ..... Restriction fragment length polymorphism
RHD ..... Rhesus blood group D antigen

RXR ..... Retinoic X receptor
SBCE ..... Single base chain extension
SCAN ..... Finger associated domain in ZNF
SM ..... Splenomegaly
SNAP ..... Soluble NSF attachment protein
SNARE ..... SNAP receptor
SNP ..... Single nucleotide polymorphism
SPM ..... Sphingomyelin
SR-BI ..... Scavenger receptor BI (also known as CLA1)
SUR1 ..... Sulfonylurea receptor 1
T ..... Thymine
Tab. ..... Table
TG ..... Trigylceride
TD ..... Tangier Disease
TDI ..... Template directed dye incorporation
T_m ..... Melting temperature
TMD ..... Transmembrane domain
UC ..... Unesterified cholesterol
USF1/2..... Upstream stimulatory factor 1 and 2
VLDL ..... Very low density lipoprotein
VNTR ..... Variable number of tandem repeats
ZNF202 ..... Zinc finger protein 202

Abbreviations for amino acids

A ..... Ala ..... Alanine
C ..... Cys ..... Cysteine
D ..... Asp ..... Aspartic Acid
E ..... Glu ..... Glutamic Acid
F ..... Phe ..... Phenylalanine
G ..... Gly ..... Glycine
H ..... His ..... Histidine
I ..... Ile ..... Isoleucine
K ..... Lys ..... Lysine
L ..... Leu ..... Leucine
M ..... Met ..... Methionine
N ..... Asn ..... Asparagine
P ..... Pro ..... Proline
Q ..... Gln ..... Glutamine
R ..... Arg ..... Arginine
S ..... Ser ..... Serine
T ..... Thr ..... Threonine
V ..... Val ..... Valine
W ..... Trp ..... Tryptophan
Y ..... Tyr ..... Tyrosine

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10 Summary

The main focus of this work was to develop high-throughput and multiplex assays for the fast and efficient analysis of sequence variations in the gene coding for the ATP-binding cassette transporter 1 (ABCA1), which has recently been identified as the main regulator of plasma HDL cholesterol.

Intensive literature and data base research was performed to library all known sequence variations in this gene. Relevant data of subjects with HDL deficiency syndrome were archived, which carry mutations in this gene. These data were stored in a web page archive (www.abca1-mutants.all.at) to provide an information resource for further research in ABCA1.

A knowledge-based sequencing approach was performed to identify novel sequence variations in ABCA1. In the promoter region, two novel VNTR polymorphisms at a ZNF202 and a SRY binding-site were identified along with three novel SNPs (G1047C, C1152T and C1440T). While the VNTR polymorphisms might not be of functional relevance, the three SNPs were found in significantly different distribution of genotypes in some cohorts and all three nucleotide exchanges appear to be associated to low HDL. Six novel sequence variations affecting amino acid sequence, including the V771M polymorphism, were found in patients with HDL deficiency syndrome and probands with aberrant HDL-levels.

LightCycler and TaqMan assays were developed for the detection of common single nucleotide polymorphisms of potential interest in the ABCA1 gene, including the V771M polymorphism. An automated high-throughput workflow with implemented TaqMan technology was established for fast and efficient genotyping of single nucleotide polymorphisms in large cohorts capable of 40,000 analyses per 24 hours. With the newly established workflow, several polymorphisms were genotyped and evaluated, including the R219K and the V771M variation. The R219K polymorphism was found in significantly increased prevalence in Italian centenarians compared to healthy Italian controls, indicating a protective effect of K219 in aging. This confirms findings from other groups concerning this polymorphism. The novel V771M polymorphism was found with significantly decreased abundance in Hungarian CAD patients compared to healthy Hungarian blood donors, indicating a protective effect of M771 on CAD. All other frequency data showed no significant difference compared to the control groups.

A multiplex assay was established for the simultaneous detection of four important polymorphisms in the ABCA1 gene using allele specific primer extension reaction for

discrimination of SNP. The method uses zip- and color-coded beads for detection with flow cytometry (Luminex). The new assay provides a fast and cost-efficient method for the determination of single nucleotide polymorphisms with reliability comparable to the well-established TaqMan technology.

Zusammenfassung

Im Mittelpunkt dieser Arbeit stand die Entwicklung von Hochdurchsatz- und Multiplex-Assays für die schnelle und effiziente Analyse von Sequenzvariationen am Modellsystem des ATP-bindenden Cassetten Transporter A1 (ABCA1)-Gens, das als wichtigster Regulator von HDL Cholesterin im Plasma identifiziert wurde.

Zunächst wurden in einer intensiven Literatur- und Datenbank-Recherche alle für dieses Gen bekannten Sequenzvariationen katalogisiert und relevante Daten von Patienten mit HDL Defizenz Syndrom, die Träger von bekannten Mutationen in ABCA1 sind, archiviert. Diese Informationen, die einer weiteren Forschung mit ABCA1 dienen sollen, wurden auf einer Internetseite (www.abca1-mutants.all.at) zusammengestellt.

Anschließend wurde ein wissensbasierter Sequenzierungsansatz durchgeführt, um gezielt neue Sequenzvariationen in ABCA1 zu finden. Hier wurden im Promotorbereich neben zwei neuen VNTR Polymorphismen auf einer ZNF202- und einer putativen SRY-Bindungsstelle, drei neue SNPs (G1047C, C1152T und C1440T) identifiziert. Während den beiden VNTR Polymorphismen eine eindeutige funktionelle Relevanz nicht nachzuweisen war, wurde bei den drei SNPs eine signifikant unterschiedliche Verteilung der Genotypen in mehreren Kollektiven festgestellt. Alle drei Einbasen-austausche scheinen mit niedrigen HDL-Werten assoziiert zu sein.

Sechs neue Sequenzvariationen die zu Aminosäureaustauschen führen (unter anderem V771M) wurden in Patienten mit HDL Defizienz Syndrom und Probanden mit anomalen HDL-Werten gefunden. Für diese und andere häufig auftretende SNPs von potentieller Relevanz wurden Assays für LightCycler und TaqMan entwickelt.

Ein automatisierter Hochdurchsatz-Workflow basierend auf TaqMan Technologie konnte erfolgreich etabliert werden, der eine schnelle und effiziente Genotypisierung von großen Patientenkollektiven (bis zu 40.000 Analysen in 24 Stunden) ermöglicht. Mit dem etablierten Workflow wurden mehrere Polymorphismen in verschiedenen Kollektiven genotypisiert, unter anderem die Aminosäureaustausche R219K und V771M. Der Austausch R219K wurde in signifikant unterschiedlicher Verteilung in italienischen über einhundertjährigen Senioren im Vergleich zu italienischen Blutspendern gefunden, was auf einen protektiven Effekt im Alterungsprozess schließen läßt. Dies ist übereinstimmend mit anderen Assoziationsstudien für diesen

Polymorphismus. Der neue Polymorphismus V771M wurde signifikant weniger häufig in ungarischen CAD Patienten im Vergleich zu ungarischen Blutspendern gefunden, was auf einen protektiven Effekt des M771-Allels in der Entwicklung von CAD schließen läßt. Alle anderen Genotypisierungen zeigten keine signifikant unterschiedliche Verteilung zwischen den einzelnen Kohorten und den Kontrollkollektiven.

Weiterhin wurde ein neues Verfahren zur multiplexen SNP Analytik auf der Basis der Luminex-Technologie etabliert. Mit Hilfe von allelspezifischer Primerextension konnten vier Polymorphismen von potentieller Relevanz simultan analysiert werden. Diese durchflusszytometrische Methode nutzt Zip-code-Technik und fluoreszenzmarkierte Beads. Das neue Assaysystem erlaubt die schnelle und effiziente Genotypisierung von SNPs mit einer Genauigkeit vergleichbar mit der anerkannten TaqMan Technologie.

11 Publications and Patents

Original publications in peer reviewed journals

M.C.O Probst, H. Thumann, C. Aslanidis, T. Langmann, C. Buechler, W. Patsch, F.E. Baralle, G.M. Dallinga-Thie, J. Geisel, C. Keller, V.C. Menys and G. Schmitz. *Screening for functional sequence variations and mutations in ABCA1*. Atherosclerosis, accepted for publication (2004).

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Review articles

M.C.O. Probst, G. Rothe and G. Schmitz, Bead-based multiplex analysis. J Lab Med 27(5/6), 182-187 (2003).

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C. Böger, M. Stubanus, M. Deinzer, M. Probst, G. Schmitz, S.R. Holmer, G.A.J. Riegger, B.K. Krämer, *Impact of genetic polymorphisms on morbidity and mortality in dialysis dependent type 2 diabetes.* Abstracts Symposium on Functional Genomics in Degenerative Diseases. Regensburg (23/05/2003).

M.C.O. Probst, C. Aslanidis and G. Schmitz, *Multiplex-DNA-Analysis Using Suspension Arrays.* Abstracts Bayern-Innovativ Kongress: Diagnostics and Therapy meets Bioinformatics and Chip Technology, Regensburg (06/03/2002).

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12 Curriculum Vitae

Mario C.O. Probst

born April 5, 1973 in Griesbach im Rottal (Germany)

09/83 – 06/92 **Comprehensive secondary school**
Major courses: mathematics, chemistry

07/92 – 09/93 **Military service**

10/93 – 08/95 **University of Regensburg (Germany)**
Study of Chemistry, basic study period

09/95 – 02/96 **University of Aberdeen (Scotland)**
Study of Chemistry (3rd year)
Erasmus exchange scheme

03/96 – 06/98 **University of Regensburg**
Study of Chemistry, advanced study period
Elective courses: Analytical Chemistry, Informatics

07/98 – 04/99 **University of Regensburg**
Institute of Analytical Chemistry, Chemo- and Biosensors
(Chair of Prof. Otto S. Wolfbeis)
Diploma thesis: "Synthesis and characterization of reactive, diode-laser-compatible fluorescent dyes"

05/99 – 08/99 **Merck & Co., Inc., Darmstadt (Germany)**
Internship in division "Pigments / Technical Chemistry"

09/99 – 12/03 **University Hospital Regensburg**
Institute of Clinical Chemistry and Laboratory Medicine
(Chair of Prof. Gerd Schmitz)
Doctoral thesis: "Development and evaluation of multiplex and high-throughput SNP analysis for the ABCA1 gene"

10/99 – 09/03 **Open University of Hagen (Germany)**
Post-graduate studies in economics for scientists and engineers
Elective courses: Production Management, Personnel Management and Organization
Diploma thesis: "Global versus local sourcing – contradictory or compatible concepts?"

04/03 – 12/03 **University of Regensburg**
Competence Center for Fluorescent Bioanalysis
Scientific project manager
Project: "Development and evaluation of a novel method for multiplex SNP analysis based on Luminex technology"

13 Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, daß ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet.

Bei der Auswahl und Auswertung folgenden Materials haben mir die nachstehend aufgeführten Personen in der jeweils beschriebenen Weise entgeltlich/unentgeltlich geholfen:

siehe „Acknowledgements“ (Seite III)

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Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Regensburg, 08. März 2004

(Mario Probst)