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Chapter 1

General Introduction

1. Isoprenoid biosynthesis pathway

1.1 Introduction

The isoprenoid biosynthesis pathway plays a central role in cellular metabolism. It provides cells with a variety of essential bioactive molecules that have pivotal functions in multiple cellular processes. Examples of these are the following non-sterol isoprenoids: 1) isopentenyl tRNAs, involved in protein translation; 2) heme A, present in the multiple heme-containing cytochrome c oxidase functioning in the mitochondrial respiratory chain; 3) dolichol, a mediator of N-linked protein glycosylation; 4) ubiquinone-10, functioning as an antioxidant and involved in electron transport in the mitochondrial respiratory chain; 5) farnesyl and geranylgeranyl moieties, used for the prenylation of proteins, which allows these to anchor in membranes. Such prenylated proteins have been implicated in signal transduction, cell cycle control, cytoskeletal organization, intracellular vesicle traffic and inflammation (1-3). The isoprenoid biosynthesis pathway also produces sterol isoprenoids among which the major sterol end product cholesterol. Cholesterol has an essential function as component of cellular membranes, but is also the precursor for steroid hormones, bile acids and oxysterols. Furthermore, it plays a crucial role in mammalian embryogenesis (4).

1.2 The mevalonate pathway

The mevalonate pathway is involved in the synthesis of all isoprenoids (fig.1) and consists of a chain of reactions in which acetyl-CoA is converted into farnesylpyrophosphate (FPP). Three acetyl-CoAs are converted into 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) in two enzyme steps. Subsequently, HMG-CoA is reduced to mevalonate by HMG-CoA reductase, the rate-limiting enzyme of the mevalonate pathway. Mevalonate is then phosphorylated twice and subsequently decarboxylated to form isopentenyl pyrophosphate (IPP). IPP and its isomer dimethylallyl pyrophosphate (DMAPP) are the five carbon (C5) building units, also called isoprene units, used for the synthesis of all isoprenoids. IPP and DMAPP are condensed in a head-to-tail configuration to form geranyl pyrophosphate (GPP). Addition of another IPP results in the formation of farnesyl pyrophosphate (FPP), the branch-point intermediate of the mevalonate pathway.

1.3 The branch point

Because FPP is the precursor for almost all isoprenoids it is also known as the branch-point intermediate of the mevalonate pathway (fig.1). FPP is the substrate for several enzymes to form the different isoprenoids. For example, FPP can be used for the farnesylation of protoheme, which is the first step in the conversion of protoheme to heme A (5) or for the farnesylation of proteins. Furthermore, addition of IPP to FPP by geranylgeranyl pyrophosphate synthase produces geranylgeranyl pyrophosphate (GGPP), which can be used for the geranylgeranylation of proteins or further elongated to nonaprenyl or decaprenyl pyrophosphate, which are precursors of the side chains in

ubiquinone. Finally, two molecules of FPP can be converted to squalene by squalene synthase, the first enzyme dedicated exclusively to the production of sterol isoprenoids (fig.1 and fig.2). To eventually produce cholesterol from squalene, a complex set of enzyme reactions is required (fig.2).

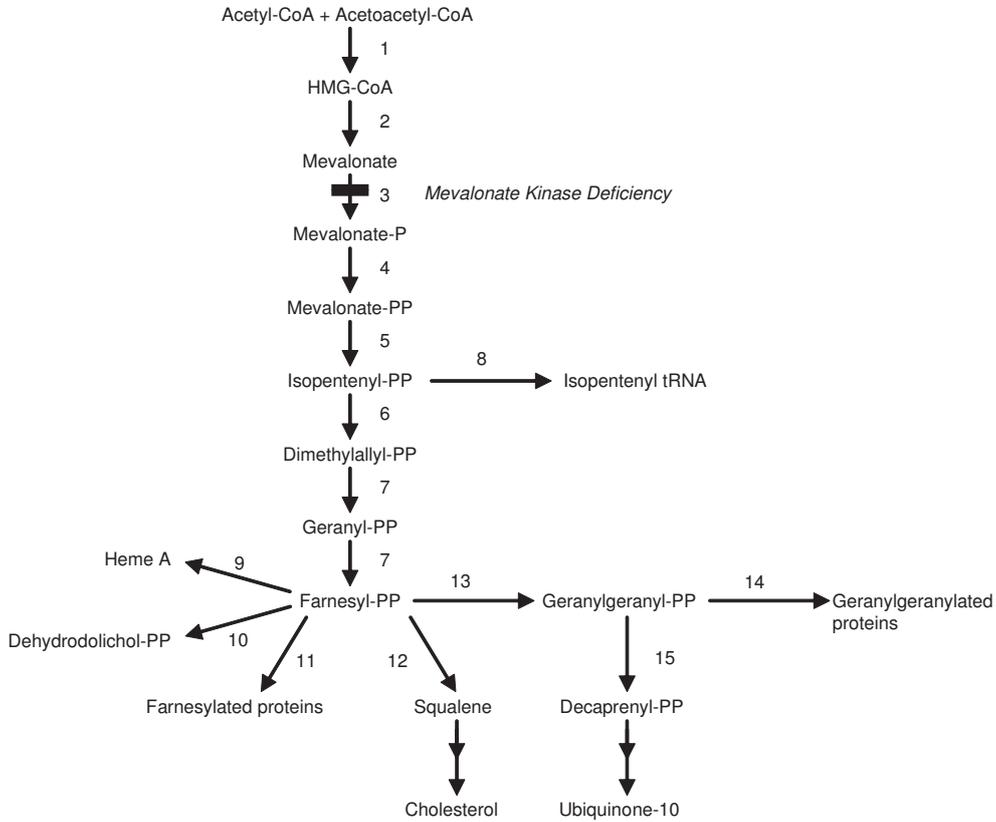


Figure 1. The mevalonate pathway and branch point

The figure includes all names of the metabolites involved. Numbering of the enzymes: 1. HMG-CoA synthase; 2. HMG-CoA reductase; 3. Mevalonate kinase; 4. Phosphomevalonate kinase; 5. Mevalonate pyrophosphate decarboxylase; 6. Isopentenyl pyrophosphate isomerase; 7. Farnesyl pyrophosphate synthase; 8. tRNA isopentenyltransferase; 9. Heme A:farnesyltransferase; 10. Dehydrodolichol pyrophosphate synthase; 11. Farnesyltransferase; 12. Squalene synthase; 13. Geranylgeranyl pyrophosphate synthase; 14. Geranylgeranyltransferase I or II; 15. Decaprenyl pyrophosphate synthase.

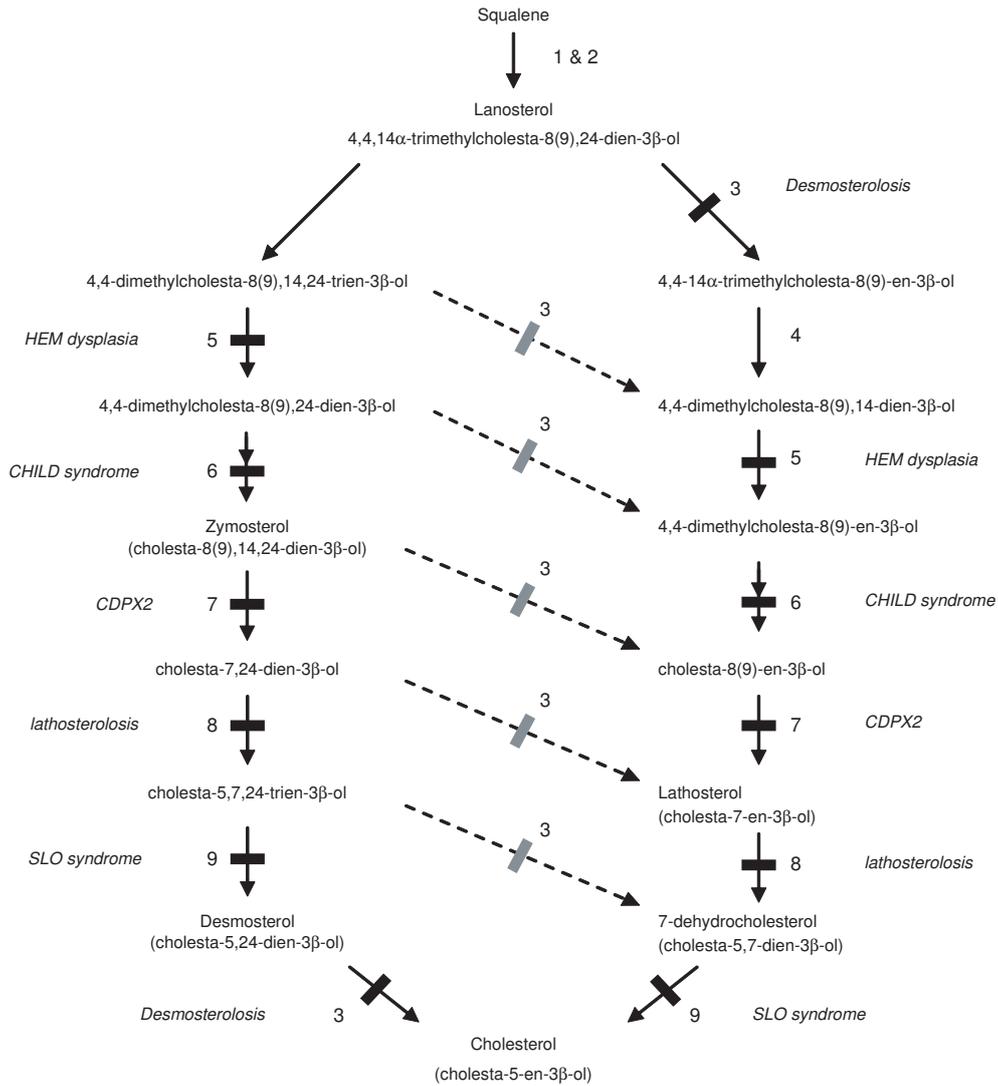


Figure 2. Cholesterol biosynthesis

For the synthesis of cholesterol two major routes have been proposed, which use the same enzymes but either postulate 7-dehydrocholesterol or desmosterol as ultimate precursor of cholesterol. The figure shows the names of the metabolites involved and the disorders linked to specific enzyme deficiencies. Numbering of the enzymes: 1. squalene epoxidase; 2. 2,3-oxidosqualene sterol cyclase; 3. sterol Δ^{24} -reductase (desmosterolosis); 4. sterol C-14 demethylase; 5. sterol Δ^{14} -reductase (HEM dysplasia); 6. sterol C-4 demethylase complex (CHILD syndrome); 7. sterol Δ^8 - Δ^7 isomerase (CDPX2); 8. sterol Δ^8 -desaturase (lathosterolosis); 9. sterol Δ^7 -reductase (SLO syndrome).

2. Regulation

2.1 SREBPs

The isoprenoid biosynthesis pathway is regulated tightly in order to produce the required amounts of sterol and non-sterol isoprenoids without risking overaccumulation of potentially toxic products, like cholesterol. End-product regulation of cholesterol levels is achieved predominantly through the activation of transcription of genes that govern the synthesis of cholesterol and its receptor-mediated uptake from plasma lipoproteins (6). This transcriptional regulation is coordinated by a family of membrane-bound transcription factors designated sterol regulatory element-binding proteins (SREBPs). SREBPs bind to sterol regulatory element 1 (SRE) in promoter regions to activate transcription when sterols are absent, but are not required for basal transcription when sterols are present (6). All genes encoding enzymes involved in the biosynthesis of cholesterol contain a sterol regulatory element 1 (SRE) in their promoter region and are subject to the feedback regulation of SREBPs (7,8). Besides regulating cholesterol biosynthesis, SREBPs can also function in the regulation of fatty acid biosynthesis, lipogenesis, and glucose metabolism (9).

In mammals there are three members of the SREBP family, SREBP-1a, SREBP-1c and SREBP-2. SREBP-1a and SREBP-1c are derived from a single gene (*SREBP-1*) through alternative splicing, whereas SREBP-2 is a product of a separate gene, *SREBP-2* (8).

SREBPs are members of the basic helix-loop-helix-leucine zipper (bHLH-Zip) family of transcription factors. Unlike other members of this family, SREBPs are synthesized as inactive precursors which localize to the endoplasmic reticulum (ER). Each SREBP precursor is organized into three domains: 1) an NH₂-terminal transcription factor domain of ~480 amino acids; 2) a middle hydrophobic region of ~80 amino acids containing two transmembrane segments; and 3) a COOH-terminal regulatory domain of ~590 amino acids (8). In order to reach the nucleus and act as a transcription factor, the NH₂-terminal domain must be released from the membrane (fig.3). SREBP cleavage-activating protein (SCAP) binds to the regulatory domain of SREBP and is both an escort protein and a sensor of sterols. Cholesterol can directly bind to the sterol-sensing domain of SCAP, inducing a conformational change of SCAP enabling the interaction with one of the two highly similar Insig proteins (10). As a result, the strong interaction of SCAP with Insig retains the SREBP-SCAP complex in the ER. When cells become depleted in cholesterol, the interaction between SCAP and Insig weakens and SCAP transports the SREBP from the ER to the Golgi (fig.3). In the Golgi, Site-1 protease (S1P) cleaves SREBP, dividing the molecule in half. Site-2 protease (S2P) then releases the NH₂-terminal transcription factor domain from the membrane. The NH₂-terminal domain, called nuclear SREBP (nSREBP), translocates to the nucleus and activates transcription by binding to the SRE of target genes (11,12) (fig.3).

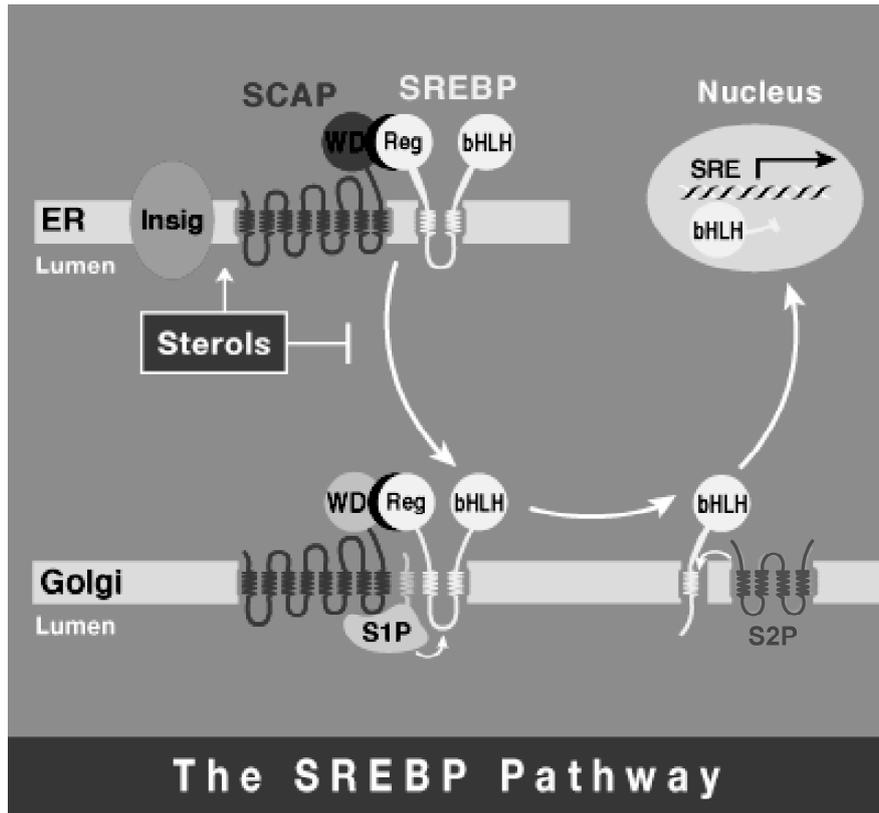


Figure 3. *SREBP activation*

Model for sterol-mediated proteolytic release of SREBPs from membranes.

Modified from ref. (9).

2.2 Posttranscriptional regulation of HMG-CoA reductase

In addition to the transcriptional regulation by SREBPs, the activity of the rate-limiting enzyme of isoprenoid biosynthesis, HMG-CoA reductase, can also be regulated by two posttranscriptional mechanisms, translational efficiency and protein degradation. The translational rate of HMG-CoA reductase mRNA is controlled by non-sterol isoprenoids. When the reductase is inhibited, its mRNA is efficiently translated in the presence of sterols, but when non-sterol requirements are satisfied by the addition of exogenous mevalonate together with sterols, translation of reductase mRNA is reduced fivefold (6). The degradation rate of HMG-CoA reductase protein is regulated by both sterol and non-sterol isoprenoids and is accelerated when sterol and non-sterol end products

accumulate in cells (6). Sever et al. reported that the Insig proteins play an important role in the sterol-accelerated degradation of HMG-CoA reductase (13). At high sterol concentrations, one of the Insig proteins and sterols bind to the sterol-sensing domain of HMG-CoA reductase, which leads to ubiquitination and proteasomal degradation of HMG-CoA reductase. Geranylgeraniol, the alcohol precursor of GGPP, can enhance this sterol-accelerated degradation of HMG-CoA reductase via Insig (14). The role for farnesol and/or FPP as a non-sterol regulator of HMG-CoA reductase degradation is still a matter of debate, however, because several reports show that farnesol can induce degradation of HMG-CoA reductase, while others show the opposite (14-19).

3. Disorders of the pathway

Several disorders of the isoprenoid biosynthesis pathway have been described. Only one disorder, mevalonate kinase deficiency, affects the biosynthesis of all isoprenoids because the deficient enzyme is located in the mevalonate pathway (fig.1). This disorder will be discussed in more detail later. Six disorders have deficiencies located in the post-squalene part of the pathway, affecting only the biosynthesis of the sterol isoprenoids (fig.2). All these disorders are characterized by multiple morphogenic, developmental and neurological abnormalities, like microcephaly, dysmorphic faces and shortening of the long bones, implying an important role for cholesterol in embryonic development.

In the most common disorder Smith-Lemli-Opitz syndrome (SLOS; MIM 270400) the enzyme 7-dehydrocholesterol reductase (7-DHCR) is deficient (20) (fig.2), resulting in elevated levels of 7- and 8-dehydrocholesterol.

Desmosterolosis (MIM 602398) is a very rare autosomal recessive disorder for which only two patients have been described with mutations in the gene coding for 24-dehydrocholesterol reductase (21-24) (fig.2).

X-linked dominant chondrodysplasia punctata (CDPX2, also called Conradi-Hunermann or Happle syndrome; MIM 302960) is a rare X-linked disorder which is usually lethal in males (25). Females have abnormal sterol profiles characterized by increased levels of 8-dehydrocholesterol and cholesta-8(9)-en-3 β -ol. Mutations have been found in the gene EBP, which encodes the sterol Δ^8 , Δ^7 -isomerase (26-28) (fig.2).

CHILD syndrome (congenital hemidysplasia with ichthyosiform erythroderma or nevus and limb defects; MIM 308050) is also an X-linked male-lethal disorder with phenotypic similarities to CDPX2, but usually with a unilateral distribution of anomalies (29). Patients can have mutations in either the EBP or the *Nsdhl* (NAD(P)H steroid dehydrogenase-like) gene. *NSDHL* encodes a sterol dehydrogenase or decarboxylase that is part of the C-4 sterol demethylase protein complex (30,31) (fig.2).

Hydrops-ectopic calcification-moth-eaten (HEM) or Greenberg skeletal dysplasia (MIM 215140) was first described by Greenberg et al. in 1988 (32). This lethal disorder

is caused by mutations in the LBR gene (33). LBR encodes the lamin B receptor, a protein which exhibits sterol Δ 14-reductase activity (fig.2).

For the last disorder, lathosterolosis (MIM 607330), two patients have been described with increased levels of lathosterol. The enzyme activity of 3-beta-hydroxy-steroid-delta-5-desaturase (SC5D) was deficient and in both patients mutations were found in the encoding SCD5L gene (34,35) (fig.2).

In addition to these sterol biosynthesis defects, there are disorders that affect the biosynthesis of only specific non-sterol isoprenoids, like heme A, ubiquinone-10 or isoprenylated proteins.

Complex IV or cytochrome c oxidase is the terminal complex of the respiratory chain. Cytochrome c oxidase deficiency (MIM 220110) can be caused by mutations in different nuclear-encoded and mitochondrial-encoded genes. One of those genes is COX10, a nuclear gene encoding heme A:farnesyltransferase (COX10), which catalyzes the farnesylation of a vinyl group resulting in the conversion of protoheme to heme O. Some of the symptoms associated with this defect are ataxia, muscle weakness, hypotonia, ptosis, pyramidal syndrome, status epilepticus, proximal tubulopathy, and mitochondrial encephalopathy (36).

Primary coenzyme Q10 (ubiquinone-10) deficiency (MIM 607426) is a subclass of respiratory chain deficiencies, due to a coenzyme Q10 biosynthesis defect. Mutations have been found in the COQ2 gene (37), coding for mitochondrial parahydroxybenzoid-polyprenyltransferase, the APTX gene (38), coding for a member of the histidine triad (HIT) superfamily, and the PDSS2 gene (39), coding for decaprenyl diphosphate synthase subunit 2. Some of the symptoms of this rare, clinically heterogeneous autosomal recessive disorder are encephalomyopathy, cerebellar ataxia, encephalopathy, renal failure, and myopathy (40-45).

Choroideremia is an X-linked chorioretinal degeneration caused by mutations in the CHM gene, which codes for the Rab escort protein-1 (REP1), a subunit of geranylgeranyl transferase-II. The deficiency causes hypogeranylgeranylation of one specific Rab protein, Rab27a, leading to progressive night blindness and loss of peripheral vision (46).

4. Mevalonate kinase deficiency

Mevalonate kinase deficiency (MKD) is an autoinflammatory disorder, belonging to a group of diseases characterized by spontaneous attacks of systemic inflammation without an apparent infectious or autoimmune etiology. Originally, based primarily on clinical presentation, 2 distinct entities associated with this defect were defined, classic mevalonic aciduria (MA) (47) and hyperimmunoglobulinemia D and periodic fever syndrome (HIDS) (48). After the discovery that both disorders are caused by a deficient activity of mevalonate kinase (MK) (47,49,50), due to mutations in the encoding *MVK* gene, they are now recognized as the severe and mild ends of the MKD spectrum.

Worldwide more than 200 patients have been diagnosed with MKD, the majority with the HIDS presentation.

Patients with the relative mild HIDS presentation suffer from recurring episodes of high fever, which occur every 2 to 6 weeks and last 3 to 7 days. These fever episodes are associated with malaise, chills, headache, arthralgias, arthritis, nausea, abdominal pain, diarrhea, skin rash, hepatosplenomegaly and lymphadenopathy (51,52). The attacks can be triggered by vaccinations, infections, surgery and physical or emotional stress, but often occur without a clear precipitating event (51).

Patients with the MA presentation experience similar fever attacks as HIDS patients, however, a clinical distinction can be made between the two presentations by the presence of congenital and developmental anomalies. Severely affected MA patients have dysmorphic features, developmental delay, hepatosplenomegaly, lymphadenopathy and anemia and they may die during early childhood. Less severely affected MA patients suffer from psychomotor retardation, myopathy, hypotonia, cataracts and cerebellar atrophy, leading to ataxia (53).

The explanation for this difference in clinical presentation lies at the biochemical level. Cells from HIDS patients still show a residual MK enzyme activity of up to 10% of the activity in controls (50,54,55). In contrast, enzyme activity in cells from patients with the MA presentation is below the level of detection (53,56). As a result of the difference in residual enzyme activity, the urinary excretion of mevalonic acid varies markedly between the two presentations. In HIDS patients, the level of mevalonic acid in urine is low to moderate (0.005 to 0.040 mol/mol creatinine) (50) and increases during fever attacks (29). In MA patients, the excretion is massive and constitutively elevated, ranging from 1 to 56 mol/mol creatinine (53). Control subjects have an excretion of less than 0.001 mol/mol creatinine.

4.1 Molecular aspects

In 1992, the cDNA clone coding for the human MK was isolated. This led to the identification of the first MA-causing mutation in the *MVK* gene, N301T (57). Transfection of the mutant cDNA construct in COS-7 cells confirmed the pathogenicity of this mutation, resulting in a severely reduced enzyme activity varying between 5 and 20% of the activity of the normal expression construct (57). The genetic basis of HIDS was not found until 1999, when two groups separately identified mutations in the *MVK* gene in HIDS patients (49,55). Since then, 63 disease-causing mutations have been identified at the cDNA and the genomic level (58-60). Most of these mutations are missense mutations, but also nonsense mutations, deletions, insertions and splicing defects have been reported. The most frequently occurring mutation in MKD is a missense mutation, a G>A transition at nucleotide 1129 changing the valine at position 377 into an isoleucine (V377I). This mutation is exclusively associated with the HIDS phenotype and is found in compound heterozygous state in the vast majority of patients with the HIDS presentation. The fact that the V377I mutation is often found in

combination with one of two other common missense mutations, H20P and I268T, which have been identified in both MA and HIDS patients indicates that the V377I mutation is responsible for the HIDS phenotype (50,61). A few patients with the HIDS phenotype have been identified with other mutations than the V377I mutation, indicating that also other mutations may cause the HIDS phenotype (49,54,55,58)

Although the V377I mutation is the most common mutation in MKD, only few patients homozygous for V377I are known. A carrier frequency study in the Dutch population showed that the predicted incidence of V377I homozygotes based on the carrier frequency of the V377I mutation is much higher than the observed incidence. This strongly suggests that homozygotes for V377I exhibit a milder phenotype of MKD or no disease-phenotype at all (62).

The disease-causing nature of the identified mutations was substantiated by characterization of mutant proteins by immunoblotting of fibroblast lysates of patients using an MK-specific antibody and heterologous expression of mutant proteins in *Escherichia coli* (*E.coli*). All mutant MK proteins expressed in *E.coli* showed markedly decreased enzyme activity (H20P, T234I, L264F, L265P, I268T, V310M and A334T) (50,56,63,64). Only the V377I mutation still had considerable residual activity when expressed in *E.coli*, much more than the residual activity measured in cells of HIDS patients. However, in fibroblast lysates of MKD patients, MK protein was hardly detectable as shown by immunoblotting (50,56). Moreover, MK protein levels could be increased when fibroblasts of MKD patients were cultured under conditions that promote a more controlled folding of precursor proteins into their mature form (i.e. growth at 30 °C or growth in the presence of the 'chemical chaperone' glycerol) (58). These observations indicate that most mutations do not directly affect MK activity but primarily affect protein folding/stability.

4.2 Immunological findings

The first description of the HIDS presentation was published in 1984, and its name was derived from the constitutively elevated levels of serum immunoglobulin (Ig)D, usually with concomitant high levels of IgA. In MA patients, an elevated serum IgD and IgA has also been reported. In contrast to the other immunoglobulin isotypes, the IgD class is mainly expressed on the surface of B lymphocytes as the major antigen receptor isotype, whereas in the serum it is found only in very low levels (65). Both IgD and IgA are probably assembled in the bone marrow, suggesting a systemic stimulation of the immune system (66). However, the physiological role of serum IgD and its role in the pathogenesis of MKD remain unclear. IgD is a potent inducer of tumour necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-1 receptor antagonist, IL-10, and IL-6 in vitro (67,68). However, its elevation in MKD is more likely to be an epiphenomenon than the cause of the inflammatory attacks, since several patients have been identified with MKD, caused by MK deficiency, but without elevated levels of serum IgD (50,69,70). Moreover, there is no relation between the level of IgD and the severity or

frequency of the attacks (51). Furthermore, other autoinflammatory diseases may have elevated IgD levels as well, like PFAPA, FMF, and BS, and various other diseases, including infections, immunodeficiencies, autoimmune and allergic diseases (68).

In patients with MKD, fever attacks are accompanied by an acute phase response, which is the production of acute phase proteins in response to inflammation. In MKD, elevated levels of C-reactive protein, serum amyloid A, soluble type II phospholipase A₂ (PLA₂), and α 1-acid glycoprotein (AGP), but also leukocytosis, neutrophilia, and steeply elevated erythrocyte sedimentation rates (ESR) (51,67,71) were found. The elevated level of AGP in patients with the HIDS presentation was not only found during attacks, but also during remissions, indicating a persistent state of inflammation (72).

Because the acute phase reaction and fever are mediated by cytokines, the production of pro- and anti-inflammatory cytokines has been studied in MKD patients. The serum levels of the pro-inflammatory cytokines interferon- γ and IL-6 rose sharply, while the level of TNF- α increased slightly during fever attacks. Also, the levels of the anti-inflammatory compounds IL-1 receptor antagonist (IL-1ra) and the soluble TNF receptors p55 (sTNFr p55) and p75 (sTNFr p75) were higher with attacks than between attacks (73). Neopterin is released by monocytes/macrophages after stimulation by IFN- γ , excreted in urine and appears to be an early and sensitive marker of activation of the cellular immune system. In patients with the HIDS presentation urinary neopterin was elevated on the first day of the fever attack and remained elevated for a few days after normalization of the body temperature (74). Plasma concentrations of IL-1 α , IL-1 β , and the anti-inflammatory cytokine IL-10 did not change during attacks (73).

Unstimulated peripheral blood mononuclear cells (PBMCs) from MKD patients obtained between attacks secrete more IL-1 β , IL-6, and TNF- α , than PBMCs from healthy subjects. Moreover, after stimulation with LPS the secretion of these pro-inflammatory cytokines increases even further (75).

In search for the pathogenesis of MKD, elevated levels of leukotriene E₄ (LTE₄) were found. The cysteinyl leukotrienes, LTC₄, LTD₄, and LTE₄, are potent lipid mediators with diverse functions in the immune and inflammatory response (76). Urinary excretion of LTE₄ is a measurement for the total systemic synthesis of cysteinyl leukotrienes in vivo (77). In patients with the MA presentation the level of urinary LTE₄ is continuously elevated (78), while HIDS patients have normal levels of LTE₄ between fever episodes and increased levels during fever episodes (79). The elevated LTE₄ in MA patients is not caused by an impaired peroxisomal degradation of cysteinyl leukotrienes. Although there is a strong positive linear relationship between urine concentrations of excreted mevalonate and LTE₄, and the levels of arachidonic acid are always elevated, the exact cause for increased urinary LTE₄ excretion is unclear (53,78).

Reactive amyloidosis is the most devastating complication in periodic fever syndromes. It is caused by accumulation of amyloid fibrils in the extracellular spaces of various organs and tissues, most notably the kidneys, liver, and spleen, resulting in

organ failure (80). One of the risk factors to develop amyloidosis is high concentrations of serum amyloid A (SAA), an acute-phase protein. Because the level of SAA is elevated during fever episodes, many patients with periodic fever syndromes develop amyloidosis. Although patients with the HIDS presentation also have elevated levels of SAA during fever episodes and even during remissions, the incidence of reactive amyloidosis is very low when compared to the other periodic fever syndromes. It is unclear why HIDS patients have such a low incidence, though it could be possible that MKD protects against amyloidosis (80).

Currently, no established treatment is available for MKD patients. In individual cases clinical improvement was achieved by the use of anti-inflammatory agents, such as corticosteroids (81), leukotriene inhibitors (81), or etanercept, a soluble TNF- α receptor (82). Moreover, a clinical trial with thalidomide had limited efficacy on the number and severity of febrile attacks (83). However, these have not led to general treatment options for MKD patients. Recently, some MKD patients have been treated with anakinra, an IL-1 receptor antagonist, which resulted in a reduction in the frequency and severity of the fever episodes (84,85). A major disadvantage of this treatment is that patients have to receive a subcutaneous injection daily. Further studies are required to confirm the clinical beneficial response of anakinra and to evaluate the effectiveness of treatment-on-demand in aborting the inflammatory attack.

4.3 Pathogenesis

The precise molecular mechanism by which a depressed MK enzyme activity leads to inflammation and fever episodes is still unknown. The symptoms in MKD patients can either be caused by an excess of mevalonic acid or a shortage of isoprenoid end products. Although there are several indications that a shortage of isoprenylated proteins causes the fever episodes, a clinical trial with simvastatin in six HIDS patients did lead to shorter periods of fever in most patients (86). Because simvastatin is an inhibitor of HMG-CoA reductase, this treatment blocks the synthesis of mevalonate and leads to a lowering of mevalonate levels. It should be noted, however, that treatment of two patients with mevalonic aciduria using lovastatin, a drug similar to simvastatin, provoked severe clinical crises in those patients (53). This discrepancy could be explained by the fact that although treatment with simvastatin results in higher residual MK enzyme activities in cells from MKD patients (87), it appears to have a negative effect on the flux towards geranylgeranyl synthesis (87,88). Therefore, the delicate balance between inhibiting HMG-CoA reductase and inducing residual MK activity could be positive in HIDS patients and negative in MA patients.

Patients with the MA presentation have decreased serum levels of ubiquinone-10 and cells from MA patients contain decreased levels of dolichol (53,89,90). However, the *de novo* biosynthesis of cholesterol and protein isoprenylation can be rather normal in fibroblasts from MKD patients when cultured under normal conditions (88,89,91). Thus, MKD cells are able to compensate for their defect in MK. This is possible

because they have increased activity of HMG-CoA reductase and the LDL receptor pathway (89,91). The increased reductase activity ensures sufficient non-sterol isoprenoid intermediates at the expense of cholesterol, and the increased expression of LDL receptors ensures sufficient uptake of LDL cholesterol, thus enabling production of non-sterol isoprenoids from mevalonate (89). Indeed, the increased HMG-CoA reductase activity is not caused by a shortage of sterol end products, because it is insuppressible by exogenous LDL cholesterol and is further upregulated under cholesterol-free culture conditions. Moreover, HMG-CoA reductase mRNA levels are normal in MA cells, indicating that the SREBP pathway, involved in transcriptional regulation, is not activated (88). Supplementation with GGOH, FOH, sterols, and mevalonate down-regulated the increased HMG-CoA reductase activity in MA cells (88). Moreover, when the increased HMG-CoA reductase activities in an MA and a familial hypercholesterolemia fibroblast cell line were compared, the suppression of the reductase activity in the MA cell line was more sensitive to supplementation with GGOH, while in the hypercholesterolemia cell line suppression of HMG-CoA reductase activity was more sensitive to a mixture of sterols (88). Together, this indicates that the regulation of the isoprenoid biosynthesis pathway is still functional in MKD and that under normal conditions shortage of one of the non-sterol end products causes the increased activity of HMG-CoA reductase.

MKD cells are able to compensate for their defect, by elevating the intracellular levels of mevalonate. However, in these cells MK has become the rate-limiting enzyme of the isoprenoid biosynthesis pathway, determining the flux through the pathway. As most mutations found in these patients have a deleterious, temperature-sensitive effect on MK protein maturation and stability, any small increase in body temperature will result in a rapid decrease in residual MK activity (61). This will lead to a rather instant disturbance of the flux through the isoprenoid biosynthesis pathway and, as a consequence, to a shortage of non-sterol end products. In response to the decreased MK activity, HMG-CoA reductase activity is increased and eventually this will re-establish normal isoprenoid production (61). However, before the flux is re-established, the production of isoprenoids is temporarily decreased. Because inhibition of geranylgeranylation of proteins increases the secretion of the pro-inflammatory cytokine IL-1 β (92,93) and addition of GGPP reduced the elevated levels of IL-1 β secretion in PBMCs from MKD patients, as well as the elevated levels of HMG-CoA reductase activity in fibroblasts from MKD patients to control levels (88,93), it is believed that the temporary shortage of one or more geranylgeranylated proteins causes the fever episodes these patients suffer from.

5. GTPases

5.1 Introduction

The small G proteins are monomeric G proteins of 20-40 kDa and are often called 'small GTPases'. More than 100 different small GTPases have been identified in eukaryotes from yeast to human. The most well-known family of small GTPases is the Ras superfamily with five major subfamilies; Ras, Rho/Rac, Rab, Sar1/ARF, and Ran. The functions of the subfamilies can be classified broadly as follows: members of the Ras subfamily mainly regulate gene expression; the Rho subfamily members (including Rho/Rac/CDC42) regulate both cytoskeletal reorganization and gene expression; the Rab and Sar1/Arf subfamily members regulate intracellular vesicle trafficking, and members of the Ran subfamily regulate nucleocytoplasmic transport during G1, S and G2 phases of the cell cycle (3).

5.2 Protein prenylation

Membrane localization is important for the function of many small GTPases and this localization is mediated by a posttranslational modification called isoprenylation. Isoprenylation is the posttranslational covalent addition of farnesyl (15-carbon) or geranylgeranyl (20-carbon) isoprenoids to cysteine residues at the carboxy terminus of proteins. The attached lipid enables membrane association of the small GTPases and, subsequently interaction with downstream effectors.

Three prenyltransferases responsible for isoprenylation have been identified, farnesyltransferase (FTase) catalyses farnesylation of proteins, while geranylgeranyltransferase-I (GGTase-I), and geranylgeranyltransferase-II (GGTase-II) catalyse geranylgeranylation (1,94). FTase and GGTase-I are both heterodimers that share an α subunit (95) and have homologous but distinct β subunits (96). Both enzymes are designated CaaX prenyltransferases since they act only on proteins containing a CaaX box at the C terminus (97). The CaaX sequence consist of an invariable cysteine, two aliphatic amino acids, and the 'X' amino acid determines which prenyl group is added. If X is Ser, Met, Ala, or Gln, the protein is processed by FTase, while Leu at this position allows modification by GGTase-I (96,97). The substrate specificity rule for FTase and GGTase-I is not absolutely stringent, with many examples of cross-prenylation. For example, when farnesylation of small GTPase K-RasB is inhibited by FTase inhibitors, it becomes a substrate for geranylgeranylation (98,99). Furthermore, RhoB can be both farnesylated as well as geranylgeranylated by GGTase-I (100).

In addition to prenylation at the cysteine residue, proteins with carboxy-terminal CaaX sequences typically undergo two further post-translational modifications, namely truncation and methylation. After prenylation, the aaX is cleaved from the s-prenyl protein by the endoprotease Rce-1 (101), resulting in a c-terminal prenylated cysteine that is carboxymethylated by an s-adenosylmethione-dependent protein carboxy-methylase (102,103).

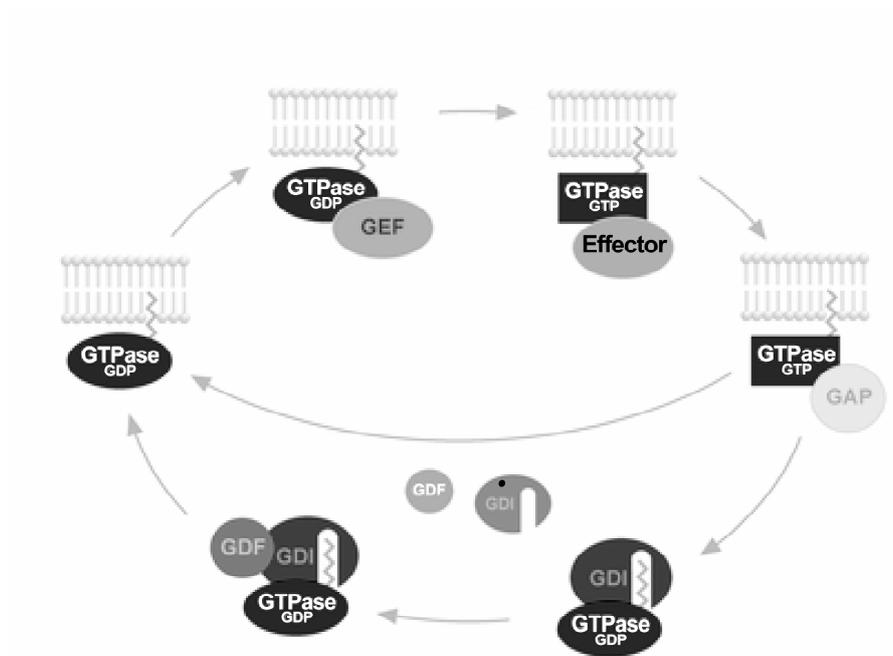


Figure 4. *GTPase activation*

Model for GTPase activation in the cell. See text for details. GEF: guanine nucleotide exchange factor, GAP: GTPase-activating proteins, GDF: GDI displacement factor. (137).

The third prenyltransferase, GGTase-II, also known as Rab geranylgeranyl-transferase, geranylgeranylates proteins that contain a C terminal sequence of Cys-Cys or Cys-X-Cys and are found exclusively in the Rab family of small GTPases. GGTase-II consists of three subunits, a Rab escort protein I (Rep1), an α - and a β -subunit. Rep1 binds unprenylated Rab proteins, presents them to the catalytic $\alpha\beta$ -subunits, and remains bound to the Rab proteins after the geranylgeranyl transfer reaction (104).

5.3 Activation

Small GTPases regulate several important cell functions, like gene expression, vesicle trafficking, cytoskeletal reorganization, nucleocytoplasmic transport, cell cycle progression, and microtubule organization. Since these processes are not constantly required in cells, they have to be switched on and off. Therefore, the small GTPases that regulate these processes function as molecular switches, cycling between an inactive GDP-bound state and an active GTP-bound state (Fig.4). An upstream signal stimulates the dissociation of GDP from the GDP-bound form, which is followed by the binding of GTP. This will eventually lead to a conformational change of the downstream effector-binding region so that this region interacts with the downstream effector. This

interaction causes the activation (or inactivation) of functions of the downstream effector. The GTP-bound form is converted to the GDP-bound form by the action of intrinsic GTPase activity, which then releases the bound downstream effector. In this way, one cycle of activation and inactivation is achieved, and small GTPases serve as molecular switches that transduce an upstream signal to a downstream effector.

To control the activation, these proteins are regulated tightly by three main classes of proteins. At first, the dissociation of GDP from the GDP-bound form is extremely slow, and therefore stimulated by a regulator, named guanine nucleotide exchange factor (GEF) (fig.4). Activation of GEF is often regulated by an upstream signal, after which it releases the bound GDP and forms a complex with the small GTPase. Subsequently, GEF is replaced by GTP to form the GTP-bound form of the GTPase. Many GEFs have been identified in humans, and most of the GEFs can activate more than one small GTPase. In addition, multiple GEFs can activate one GTPase (105,106). The GDP/GTP-exchange reaction is thought to be the rate-limiting step in the GTP-binding/GTP hydrolytic cycle of GTPases and the GEFs have therefore been proposed as key regulators of the small GTPases.

Small GTPases themselves can hydrolyse GTP to GDP. However, the GTPase activity of small GTPases is very slow and is therefore accelerated by a group of regulatory proteins known as GTPase-activating proteins (GAPs) (fig. 4). GAPs are classified according to their GTPase subfamily (Ras-GAP, Rho-GAP, Rap-GAP, etc) with sequence homology within subfamilies but not between families (107).

The third group of regulatory proteins of the small GTPases are the guanine nucleotide dissociation inhibitors (GDIs). GDIs bind to the carboxyl terminus of small GTPases in a 1:1 ratio to prevent activation. To date, only four human GDIs have been identified, three RhoGDIs and a RabGDI that has a different structure but performs the same function for proteins in the Rab family (108-110). The human RhoGDIs are the ubiquitously expressed RhoGDI (or RhoGDI α / RhoGDI1) (111,112), the haematopoietic cell-selective Ly/D4GDI (or RhoGDI β / RhoGDI2) (113), and Rho-GDI γ (or RhoGDI3), which is preferentially expressed in brain, pancreas, lung, kidney, and testis (114,115). RhoGDIs can prevent activation of Rho GTPases via three distinct mechanisms. First, they maintain Rho GTPases as soluble cytosolic proteins by forming high-affinity complexes in which the isoprenoid moiety of the GTPase is shielded from the solvent by its insertion into the immunoglobulin-like hydrophobic cavity of the GDI (116-119). Second, RhoGDIs inhibit the dissociation of GDP from the Rho proteins, maintaining the GTPases in an inactive form and preventing GTPase activation by GEFs. Interaction of GDIs with the side chain of the essential Thr35 residue in switch I of the GTPase stabilizes Mg²⁺ coordination, thereby stabilizing nucleotide binding and preventing the GEF-catalysed nucleotide exchange reaction (117,118). Finally, the GDIs are able to interact with the GTP-bound form of the GTPase to prevent interactions with effectors (109,110). Because of these mechanisms, GDIs are major regulators of Rho GTPase activity and function. However, GDIs themselves are also

regulated by other proteins, called GDI displacement factors (GDFs) (fig. 4). Members of the ERM (ezrin/radixin/moesin) family, like a number of other proteins and lipids (reviewed in ref. (109,120)), have been described to induce the release of RhoA from RhoGDI (121). Moreover, it has been proposed that the activity of kinases that modulate the phosphorylation state of RhoGDIs might also act in regulating activation of the GDIs (109). However, further studies on these pathways have to be done.

5.4 Functions and diseases

Small GTPases participate in a multitude of cellular functions. The functions of farnesylated proteins from the Ras subfamily in gene expression are well characterized, however, the focus here will be on the functions of geranylgeranylated GTPases. Rho, Rac, and CDC42 of the Rho GTPases are the most extensively studied geranylgeranylated proteins. The major function of the Rho GTPases is the regulation of the assembly and organization of the actin cytoskeleton. As reorganization of the cytoskeleton plays a crucial role in many cellular functions, Rho/Rac/CDC42 proteins indirectly play a role in the regulation of cytokinesis, phagocytosis, pinocytosis, cell adhesion, cell migration, morphogenesis, and axon guidance (122).

In addition, other functions of the Rho/Rac/CDC42 proteins have been elucidated. Rac protein is very important for the function of NADPH oxidase. NADPH oxidase produces reactive oxygen species (ROS) to kill and degrade bacteria that are phagocytosed by leukocytes. It is a multicomponent enzyme consisting of a membrane-associated catalytic moiety and cytosolic regulatory proteins. Upon stimulation, the cytosolic proteins, p47^{phox}, p67^{phox}, p40^{phox} and Rac GTPase are translocated to the membrane to form the enzyme complex. It is not clear if Rac is solely an adaptor that positions the cytosolic proteins at the membrane component or that Rac itself also regulates the electron transfer reactions (123). That NADPH oxidase enzyme activity is essential for host defence is stressed by the disorder chronic granulomatous disease (CGD), caused by mutations in one of the subunits gp91^{phox}, p22^{phox}, p47^{phox}, and p67^{phox}. Patients with CGD are unable to kill phagocytosed bacteria and fungi and therefore suffer from chronic and sometimes life-threatening bacterial and fungal infections, resulting in lymphadenitis, granuloma formation, abscesses, and sepsis (124,125). Another disorder, neutrophil immunodeficiency syndrome, is caused by mutations in Rac2 (126). Only one patient has been described with neutrophil immunodeficiency syndrome, who suffered from severe recurrent bacterial infections and poor wound healing (127), due to a heterozygous missense mutation, resulting in the substitution of an asparagine for aspartate. This aspartate residue binds the catalytic Mg⁺⁺, which is required for GTP hydrolysis and Rac2 function. The mutant protein is unable to bind GTP and is suggested to affect the stability of the normal Rac2 protein, because of diminished Rac2 levels in the patient (126).

Rho, Rac and CDC42 are also able to regulate gene expression (3). Rac and CDC42 are activators of the Jun NH2-terminal kinase (JNK) cascade and p38 MAP

kinase (128,129). Moreover, Rho, Rac and CDC42 have been reported to regulate transcriptional activation by serum response factor (SRF) (130). The activation of the MAPK signalling pathways is independent of changes to the actin cytoskeleton, however, the activation of SRF appears to be directly linked to the levels of unpolymerized actin (131). Rho, Rac, and CDC42 have also been shown to activate the transcription factor NF- κ B (132).

Another subfamily of geranylgeranylated proteins are the Rab GTPases. Posttranslationally modified Rab GTPases appear to be targeted to distinct intracellular compartments and thus display a characteristic pattern of subcellular localization. Rab proteins function in the tethering/docking of vesicles to their target compartment, leading to membrane fusion. Moreover, Rab proteins have been shown to determine the distribution of cellular compartments by regulating the movement of vesicles and organelles along cytoskeletal filaments (133). Although more than 60 human RAB genes are known, deficiency of only one Rab protein is implicated in a human disease. Mutations in the RAB27A gene causes the rare autosomal recessive disorder, Griscelli syndrome (GS). Patients with GS have an immunodeficiency due to failure of cytotoxic T lymphocytes to secrete their lytic granules, and albinism due to defects in melanosome transport. Most patients develop an uncontrolled T lymphocyte and macrophage activation syndrome (hemophagocytic syndrome) (134).

Although there are some studies that show that unprenylated proteins may also have functional effects (135,136), for most small GTPases isoprenylation is critical for intracellular trafficking and function, as several studies have shown that prenylation is necessary for the small GTPases to interact with their regulatory proteins. Therefore, inhibition of the isoprenoid biosynthesis pathway and subsequent protein prenylation will disturb proper functioning of the small GTPases.

6. Outline of this thesis

MKD is caused by mutations in the *MVK* gene. Up till now, 63 disease-causing mutations have been identified at the cDNA and genomic level. Most of these mutations do not directly affect MK activity but primarily affect protein folding/stability (chapter 2). The residual MK enzyme activity in MK-deficient cells is very low, however, the biosynthesis of cholesterol can be rather normal in these cells. This is due to the fact that MK-deficient cells are able to compensate for their deficiency by elevating HMG-CoA reductase enzyme activity. In chapter 3 we show that the increased activity of HMG-CoA reductase is caused by shortage of one of the non-sterol end products. Because MK has become the rate-limiting enzyme in the isoprenoid biosynthesis pathway in MK-deficient patients, it determines the flux through the pathway. Manipulation of isoprenoid biosynthesis can increase the residual MK enzyme activity and may be an interesting therapeutic option to treat MK deficiency (chapter 4). Although the deficient MK enzyme activity in principle affects the biosynthesis of all

isoprenoids, there are indications that in particular a temporary shortage (or dysfunction) of one or more geranylgeranylated proteins is responsible for the fever episodes in these patients. Because small GTPases highly depend on geranylgeranylation for proper functioning, we studied the effect of MK deficiency on the geranylgeranylation and activation of two small GTPases, RhoA and Rac1 in chapter 5.

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