Biochemical and genetic aspects of mevalonate kinase and its deficiency
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Chapter 1

General Introduction
1. **Biosynthesis of isoprenoids**

1.1. **Isoprenoid biosynthesis**

Isoprenoids make up a large group of essential compounds involved in diverse cellular processes. Among these are: (1) ubiquinone-10, which functions as an antioxidant and plays an essential role in the electron transport in the mitochondrial respiratory chain; (2) heme A, as present in the multiple heme-containing cytochrome c oxidase also functions in the mitochondrial respiratory chain; (3) dolichol, a mediator of N-linked protein glycosylation; (4) isopentenyl tRNAs, involved in protein translation; and (5) isoprenylated proteins. Isoprenylation is the post-translational covalent addition of farnesyl-and geranylgeranylmoieties to proteins, which in most cases makes them membrane associated. Many isoprenylated proteins participate in important cellular functions, such as signal transduction, cell cycle control, cytoskeletal organization and intracellular vesicle traffic [1].

In addition to these non-sterol isoprenoid compounds, cells produce sterols such as cholesterol. Cholesterol functions not only as a lipid in cellular membranes, but also as the precursor for steroid hormones, bile acids and oxysterols. Furthermore, cholesterol can be covalently linked to the hedgehog class of embryonic signaling proteins, which function in embryonic tissue patterning [2]. All isoprenoids contain one or more (modified) C5 isoprene units. This C5 isoprene unit is synthesized in the mevalonate pathway from acetyl-CoA.

1.2. **The mevalonate pathway**

In all metazoan organisms isoprenoids are made via the mevalonate pathway. This pathway starts with three acetyl-CoAs which are converted into 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) in two enzyme steps (figure 1)[3]. HMG-CoA is the substrate of the rate-limiting enzyme of the pathway, the highly regulated HMG-CoA reductase (HMGR) which produces mevalonate [4]. Subsequently, mevalonate is phosphorylated twice, which yields 5-pyrophosphomevalonate. Decarboxylation of this compound gives isopentenyl pyrophosphate (IPP), a C5 isoprene unit. After isomerization to dimethylallyl pyrophosphate (DMAPP), a head-to-tail condensation of IPP to DMAPP results in the formation of geranyl pyrophosphate (GPP). Addition of another IPP gives farnesyl pyrophosphate (FPP), the branch-point metabolite. Another fate of IPP is its addition to an adenosine in some tRNAs.

1.3. **The branch-point enzymes and cholesterol biosynthesis**

FPP is the precursor for almost all isoprenoids and therefore is the substrate for all branch-point enzymes (figure 2). The second substrate varies and can be either a protein, heme A, IPP or FPP. Addition of one IPP to FPP gives geranylgeranylpyrophosphate (GGPP) that can either be used directly for geranylgeranylation of proteins or further elongated to nonaprenyl or decaprenyl pyrophosphate. These molecules are used for the biosynthesis of ubiquinone-9 in rats and mice or ubiquinone-10 in humans, which contain 9 or 10 isoprene units, respectively. In contrast to these trans-prenyltransferases (E-prenyltransferases), dehydrodolichol pyrophosphate synthase, involved in dolichol synthesis, is a cis-prenyltransferase (Z-prenyltransferase)[5].
Figure 1. The mevalonate pathway. The figure includes all the structures and names of the metabolites involved. The different enzymes involved are numbered as follows: 1. Acetoacetyl-CoA thiolase (Acetyl-CoA:C-acetyltransferase, EC 2.3.1.9); 2. 3-Hydroxy-3-methylglutaryl-CoA synthase (HMGS, EC 4.1.3.5); 3. 3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR, EC 1.1.1.34); 4. Mevalonate kinase (MK, EC 2.7.1.36); 5. Phosphomevalonate kinase (PMK, EC 2.7.4.2); 6. Mevalonate pyrophosphate decarboxylase (MPD, EC 4.1.1.33); 7. Isopentenyl pyrophosphate isomerase (IPPI, EC 5.3.3.2); 8. Farnesyl pyrophosphate synthase (FPPS, Geranyltransferase, EC 2.5.1.10); 9. tRNA isopentenyltransferase (EC 2.5.1.8).

Three different protein prenyltransferases have been identified. Protein farnesyltransferase (FTase) is related to protein geranylgeranyltransferase I (GGTase I): both are heterodimers sharing a common α-subunit. The β-subunits are different but share significant sequence similarity. Protein substrates for FTase are Ras proteins and nuclear lamins, whereas GGTase I uses Rac, Rho and most γ-subunits of heterotrimeric G proteins. Geranylgeranyltransferase II (GGTase II) is also known as Rab geranylgeranyltransferase, and geranylgeranylates proteins that belong to the Rab protein family [1].

Condensation and reduction of two of FPP molecules by squalene synthase yields squalene, a C30 molecule (6 isoprene units) and the first committed intermediate in the production of the sterol isoprenoids (figure 3). Following the formation of lanosterol (4,4,14 α-trimethylcholesta-8(9),24-dien-3β-ol) by cyclization of squalene, a series of enzyme reactions is required to eventually produce cholesterol. These include one demethylation at C-14 and two demethylations at C-4, three reductions of the Δ14, Δ7 and Δ24 double bonds, one isomerization of Δ8 to Δ7 and one desaturation between C-5 and C-6. Although the various enzyme reactions required for the conversion of lanosterol to cholesterol have been established, their preferred sequence may vary depending on the tissue in which they occur. Alternative routes have been proposed which, dependent in particular on the timing of reduction of the Δ24 double bond, postulate either 7-dehydrocholesterol (cholesta-5,7-dien-3β-
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ol) or desmosterol (cholest-5,7,24-tien-3β-ol) as the ultimate precursor of cholesterol [6-9]. It is assumed that the enzymes involved are rather non-specific and can handle various intermediates. Alternatively, the substrate specificity may vary between tissues in which cholesterol biosynthesis occurs or/and the relative activities of the different enzymes are regulated by intracellular factors that operate in different tissues [9].

Figure 2. The branch-point enzymes. The figure includes all end-products which can arise from the branch-point metabolite, FPP. The different enzymes involved are numbered as follows: 1. Heme A:farnesyltransferase; 2. Geranylgeranyl pyrophosphate synthase (GGPPS, Farnesyltransf erase, EC 2.5.1.29); 3. Squalene synthase (Farnesyl diphosphate farnesyltransferase, EC 2.5.1.21); 4. Decaprenyl pyrophosphate synthase (trans-Nonaprenyltransferase, EC 2.5.1.11); 5. Decaprenyl-4-hydroxybenzoate transferase; 6. Dehydrodolichyl pyrophosphate synthase (cis-Prenyltransferase); 7. Protein farnesyltransferase (FTase); 8. Protein geranylgeranyltransferase I (GGTasel); 9. Protein geranylgeranyltransferase II (GGTaseII).

1.4. Metabolism of farnesol and geranylgeraniol

Farnesol (FOH) and geranylgeraniol (GGOH) are no direct intermediates in the biosynthesis of isoprenoids, however, both compounds can be utilized by cells for isoprenoid biosynthesis when added to the culture medium [10-12]. For example, FOH and GGOH can rescue farnesylation and geranylgeranylation of proteins and cell cycle progression, when HMG R is blocked by the addition of statins [13]. On the other hand, FOH is an intermediate in the degradation of superfluous FPP and has been implicated to be a post-transcriptional, non-sterol regulator of HMG R since it accelerates the turnover of the HMG R protein [14-20]; however, this is still a matter of debate [21]. In addition, exogenous FOH and/or GGOH have an effect on several other physiological processes, including inhibition of phosphatidylcholine biosynthesis [22, 23], induction of apoptosis, inhibition of cell cycle progression and actin cytoskeletal disorganization [22, 24].

FOH and GGOH cannot be used directly for isoprenoid biosynthesis, but have to be ‘activated’ to their pyrophosphates, FPP and GGPP. The enzymes involved have been characterized at the biochemical level and it was shown that FOH and GGOH are phosphorylated by two successive monophosphorylation reactions [25]. The first step appears aspecific for added nucleotides, whereas the second step is performed by a CTP-specific kinase [25-27].

Degradation of FPP also occurs. This was noted when potent squalene synthase inhibitors (zaragozic acids) were isolated and characterized [28]. As statins, these inhibitors could be useful as lipid lowering drugs. When used in a trial, individuals excreted several novel dicarboxylic acids in urine, identified as specific breakdown products of FPP (farnesol-derived dicarboxylic acids)[29]. In cells, excess FPP is converted into farnesol by farnesyl
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pyrophosphatase [18]. Farnesol is oxidized to farnesoic acid and in the liver via ω- and β-oxidation converted into several dicarboxylic acids, which can be excreted in urine.

The physiological relevance of these ‘salvage’ and ‘degradation’ pathways remains to be demonstrated.

Figure 3. Biosynthesis of cholesterol. For this pathway two main alternate routings have been proposed, which use the same enzymes but either postulate 7-dehydrocholesterol or desmosterol as ultimate precursor of cholesterol. The figure includes structure and name of the metabolites involved. The different enzymes involved are numbered as follows: 1. Squalene monooxygenase (Squalene epoxidase, EC 1.14.99.7); 2. Lanosterol synthase (EC 5.4.99.7); 3. 3β-hydroxysterol Δ7-reductase (desmosterol reductase); 4. cholesterol C-14 demethylase (CYP51); 5. 3β-hydroxysterol Δ5-reductase; 6. cholesterol C-4 demethylase; 7. 3β-hydroxysterol-Δ8, Δ7-isomerase; 8. 3β-hydroxysterol Δ9-desaturase (lathosterol dehydrogenase); 9. 3β-hydroxysterol Δ7-reductase (7-dehydrocholesterol reductase, 7-DHCR, EC 1.3.1.21).
2. Regulation of the mevalonate pathway

As for most anabolic pathways, isoprenoid biosynthesis is regulated tightly in order to allow a constant production of the multiple isoprenoid compounds and to avoid overaccumulation of toxic intermediates or products, such as cholesterol [4]. Feedback regulation of isoprenoid biosynthesis by cholesterol is achieved predominantly through repression of transcription of genes that govern the synthesis of cholesterol (HMGS and HMGR) and its receptor-mediated uptake as plasma lipoproteins (LDL-receptor) [4]. The promoters of these genes were shown to contain a 10 bp cis element that was named sterol regulatory element 1 (SRE-1). These SREs consist of a direct repeat of 5'-PyCAPy-3' and function as conditional positive elements that enhance transcription when sterols are absent, but they are not required for basal transcription when sterols are present [4]. The transcription factors that bind to the SREs are called sterol regulatory element binding proteins (SREBPs) and belong to the family of basic helix-loop-helix leucine zipper transcription factors. Two genes, SREBP-1 and SREBP-2, encode for three SREBP proteins, SREBP-1a, SREBP-1c and SREBP-2. SREBP-1a and SREBP-1c originate from SREBP-1, as a result from alternative transcription initiation start sites and alternative splicing [30]. The three different proteins can be activated preferentially and function not only in regulation of isoprenoid biosynthesis, but also of fatty acid biosynthesis, lipogenesis and glucose metabolism; SREBP-2 activates genes involved in cholesterol biosynthesis better than genes involved in fatty acid biosynthesis, whereas the opposite is true for SREBP-1a. SREBP-1c has a preferential role in the activation of genes involved in lipogenesis and glucose metabolism [31-33].

SREBPs are synthesized as ~125-kDa proteins that are inserted into the membrane of the nucleus and endoplasmic reticulum (ER). All SREBPs share a similar tripartite structure consisting of: (1) an amino-terminal transcription factor domain; (2) a middle hydrophobic region with two transmembrane segments; and (3) a carboxy-terminal regulatory domain [30]. These proteins are much larger than the initially purified ~68 kDa proteins from nuclear extracts, indicating that the SREBPs are proteolytically processed [31]. The elegant and elaborate mechanism by which this happens explains how SREBPs function and has been studied most extensively for SREBP-2 [30]. This mechanism involves another protein called SREBP-cleavage activating protein (SCAP), which interacts with the regulatory domain of SREBP. This protein has a so-called ‘sterol-sensing domain’ that also has been identified in other proteins such as HMGR. In the absence of sterols, SCAP functions as a chaperone protein and transports the SREBP to the Golgi. In this compartment, SREBP is cleaved in two subsequent steps by proteases called Site-1 and Site-2 protease (S1P and S2P). This proteolysis releases a 68 kDa fragment that enters the nucleus and activates target genes [34-36]. Besides by cholesterol, SREBP processing is also inhibited by oxysterols, unsaturated fatty acids and polyunsaturated fatty acids (PUFAs) [31-33]. Insulin promotes expression and cleavage of SREBP1c [31-33].

There is substantial evidence that all enzymes of isoprenoid and cholesterol biosynthesis are under coordinate regulation by SREBPs, since overexpression of SREBP-1a or SREBP-2 in mice induces expression of every enzyme involved in isoprenoid and cholesterol biosynthesis [37]. Furthermore, when rats were fed diets with bile acid sequestrants and/or statins, there was an increase in the activities of HMGR, MK, PMK and IPP1 [38-41].

The increases in HMGR activity can be much higher than its increase in mRNA level. Statins for example can induce a 200-fold increase in HMGR protein, whereas mRNA levels
are elevated only 8-fold [4]. This can be explained by multilevel regulation of this enzyme. In addition to the regulation at the transcriptional level, HMGR activity is regulated also by three post-transcriptional mechanisms. These include translational efficiency of the HMGR mRNA, turnover of the HMGR protein and phosphorylation of the HMGR protein [4]. The rate of translation of HMGR mRNA is dictated by the cells’ demand for non-sterol isoprenoids. When mevalonate production is blocked by statins, the mRNA is efficiently translated even in the presence of sterols, but when also the non-sterol requirements are satisfied by the addition of mevalonate, the translation-rate reduces 5-fold [42]. The degradation rate of the protein is governed by both sterol and non-sterol isoprenoids and can be accelerated 5-fold [37, 43]. The sterols probably act via the membrane-spanning domain of HMGR, which is not necessary for catalytic activity, but also has a ‘cholesterol sensing domain’ [4]. FOH is most probably a non-sterol regulator of HMGR degradation [14-20]. Thus, the combined action of these two mechanisms together with the transcriptional regulation can induce an impressive adaptive response.

In addition, the catalytic activity of HMGR can be decreased by reversible phosphorylation of a serine by the AMP-activated protein kinase. The same enzyme also inactivates acetyl-CoA carboxylase, the rate-limiting enzyme in fatty acid biosynthesis. This AMP-activated protein kinase functions as a metabolic sensor or ‘fuel gauge’ that monitors cellular energy levels. The enzyme becomes activated when cellular ATP levels drop and switches off ATP-consuming anabolic pathways and switches on ATP-producing catabolic pathways [44].

3. Subcellular localization

For a long time isoprenoid biosynthesis had been believed to take place in the endoplasmic reticulum (ER) and cytosol. Enzymes with soluble substrates would reside in the cytosol, whereas the enzymes handling lipophilic substrates would be present in the ER membrane. The only significant exception to this is HMGR, which is an integral membrane protein of the ER, but has its catalytic domain localized in the cytosol. At this moment, however, there is some evidence that also peroxisomes are important for the biosynthesis of isoprenoids (for reviews see [45-47]) . Peroxisomes are organelles with a variety of functions in cellular metabolism. These include among others, β-oxidation of fatty acids and fatty acid derivatives, ether-phospholipid biosynthesis, α-oxidation and polyunsaturated fatty acid biosynthesis [48]. The studies on the role of peroxisomes in isoprenoid biosynthesis were initiated after the finding of HMGR in the matrix of peroxisomes by immuno-electron microscopy [49]. In addition, there are biochemical studies showing that peroxisomes have all the enzymes necessary for the conversion of acetyl-CoA to FPP, dolichol and cholesterol [50-62]. Furthermore, the cloning of the genes encoding the enzymes of the mevalonate pathway revealed several potential peroxisomal targeting sequences (PTS1 or PTS2), which may direct proteins via the PTS1 or PTS2 receptor protein (PEX5 and PEX7) to the protein import machinery of the peroxisome. This enabled molecular biological studies showing functionality of these targeting sequences [39, 41, 63, 64].

Also, in patients suffering from peroxisomal biogenesis disorders, abnormally low plasma cholesterol concentrations have been reported [65-69], suggesting a role for this organelle in isoprenoid biosynthesis (for reviews on peroxisome biogenesis see [70, 71], for a review on peroxisomal disorders see [72]). In these disorders, which include Zellweger syndrome and rhizomelic chondrodysplasia punctata (RCDP) type 1, peroxisomal enzymes
become mislocalized to the cytosol, due to a defect in the peroxisomal protein import machinery. In many cases this leads to degradation and/or inactivation of these enzymes, with a deficient activity as a result. This phenomenon has been reported also for several of the enzymes involved in isoprenoid biosynthesis, however, the findings in literature are conflicting [56, 73-75]. We have shown recently that at least part of the observed deficient activities in these disorders are the result of the bad condition of the analyzed material rather than the result of mislocalization [76]. It can be concluded that the absence of functional peroxisomes does not lead to a deficiency of enzymes involved in cholesterol biosynthesis and that intact peroxisomes are not a prerequisite for isoprenoid biosynthesis [76, 77]. The reduced cholesterol levels may be an epiphenomenon of peroxisomal biogenesis disorders. Since these results do not exclude a peroxisomal localization under physiologic circumstances, conclusive evidence on the role of peroxisomes in isoprenoid biosynthesis is still lacking.

4. Disorders of isoprenoid biosynthesis
Most disorders of isoprene biosynthesis occur in the postsqualene part of the pathway and affect only the biosynthesis of cholesterol. In line with the important role of cholesterol in embryogenesis, these syndromes are characterized by multiple developmental abnormalities. Other disorders affect the biosynthesis of heme A, ubiquinone-10 or some isoprenylated proteins. Only one disorder affects the biosynthesis of all isoprenoids since the deficiency is located in the mevalonate pathway. A short description of each of these syndromes is given below.

4.1. Smith-Lemli-Opitz syndrome (MIM 270400)
Smith-Lemli-Opitz syndrome (SLOS) was described already in 1964 [78]. It is a disorder characterized by multiple morphogenic and congenital abnormalities. The most prominent are microcephaly, short nose with anteverted nares, cleft palate, micro- or retrognatia, syndactyly of the 2nd and 3rd toe and postaxial polydactyly. Furthermore, most patients have feeding difficulties and psychomotor retardation [79]. The biochemical basis of SLOS was solved in 1993 with the finding of abnormally low levels of cholesterol in plasma and tissues of patients [80, 81]. In conjunction, elevated concentrations of 7- and 8-dehydrocholesterol were found, strongly suggesting a deficiency in 7-dehydrocholesterol reductase (7-DHCR) activity, which was confirmed later [82, 83]. In 1998, three groups cloned the cDNA encoding the human 7-DHCR, after which the molecular basis of SLOS was solved by the finding of mutations in this gene (DHCR7) [84-87].

4.2. Desmosterolosis (MIM 602938)
Desmosterolosis is a very rare inherited disorder of cholesterol biosynthesis, with only two reported patients [88-90]. Although one patient has macrocephaly instead of microcephaly, both patients have phenotypic similarities with SLOS. The cholesterol concentration was low in plasma and tissues of these patients. Instead of 7-dehydrocholesterol, these patients accumulated 24-dehydrocholesterol, which suggested a deficiency in 24-dehydrocholesterol reductase (DHCR24) activity. Indeed, in both patients mutations were identified in the encoding gene (DHCR24)[90].
4.3. **X-linked dominant chondrodysplasia punctata (MIM 302960)**
X-linked dominant chondrodysplasia punctata (CDPX2), also called Conradi-Hünermann or Happle syndrome is a sporadic, X-linked disorder which is lethal in males [91]. Female patients clinically present with skeletal and skin abnormalities. The skeletal abnormalities include chondrodysplasia punctata (epiphysic stippling) and shortening of the long bones. Both symptoms are found also occasionally in SLOS [92]. The abnormalities in this syndrome occur bilateral and asymmetric. Patients have increased levels of 8-dehydrocholesterol and cholesta-8(9)-en-3β-ol, suggesting a deficiency of 3β-hydroxysterol Δ^8, Δ^7-isomerase. Mutations in CDPX2 patients have been found in the emopamil binding protein gene (EBP), which encodes the sterol Δ^8, Δ^7-isomerase [92-94].

4.4. **CHILD syndrome (MIM 308050)**
The symptoms in CHILD syndrome (congenital hemidysplasia with ichthyosiform erythroderma and limb defects) resemble those in CDPX2, however, they occur unilateral, most frequently affecting the right side of the body [95]. Patients can have mutations in either the EBP or the Nsdhl (NAD(P)H steroid dehydrogenase-like) gene. The Nsdhl gene encodes an enzyme which is part of the cholesterol C-4 demethylase complex [96, 97].

4.5. **Hydrops-ectopic calcification-moth eaten skeletal dysplasia (MIM 215140)**
Hydrops-ectopic calcification-moth eaten (HEM) skeletal dysplasia, or Greenberg dysplasia, is a disorder characterized by short-limb dwarfism, an unusual ‘moth-eaten’ appearance of the markedly shortened long bones, bizarre ectopic ossification centers and a marked disorganization of chondroosseous histology [98]. Patients accumulate sterols with a Δ^14 double bond, suggesting a deficiency of the sterol Δ^14-reductase [99]. Two genes have been reported with sterol Δ^14-reductase activity, the lamin B receptor and the TM7SF2 gene. No mutations have been identified in the TM7SF2 gene of 5 patients [99].

4.6. **Respiratory chain deficiencies**
Cytochrome c oxidase (COX) is the terminal complex in the mitochondrial respiratory chain. Isolated COX deficiency (MIM 220110) is the most frequent cause of respiratory chain defects in humans and can be caused by mutations in mitochondrial or nuclear genes. In one family, mutations were found in the nuclear gene that encodes heme A:farnesyltransferase (COX10). Some of the symptoms are mitochondrial encephalopathy, ataxia, muscle weakness, hypotonia, ptosis, pyramidal syndrome, status epilepticus and proximal tubulopathy [100].

Ubiquinone-10 deficiency is a subclass of respiratory chain deficiencies most probably due to an ubiquinone-10 biosynthesis defect. Patients are characterized by severe encephalopathy and renal failure, but can be treated with ubidecarenone [101].

4.7. **Deficiencies of protein prenylation**
Choroideremia (MIM 303100) is an X-linked chorioretinal degeneration characterized by progressive night blindness and loss of peripheral vision invariably resulting in complete blindness. Mutations in the Rab escort protein 1 (REP1), cause hypogeranylgeranylation of one specific Rab protein (Rab27a), which is highly expressed in the affected layers of the eye [102].
Griscelli syndrome (MIM 214450) is an autosomal recessive disorder caused by mutations in the \textit{MYO5A} or \textit{RAB27A} gene. Patients have an immunodeficiency due to failure of cytotoxic T lymphocytes to secrete their lytic granules, and albinism due to defects in melanosome transport. These symptoms are in accordance with the role of Rab proteins in traffic between cellular compartments. Most patients develop an uncontrolled T lymphocyte and macrophage activation syndrome (hemophagocytic syndrome) [102].

A mutation in the \(\alpha\)-subunit of GGTase II has been described in the \textit{gunmetal} mouse. This spontaneous, recessive mutation leads to reduced geranylgeranylation and membrane association of Rab proteins. The \textit{gunmetal} mice exhibit prolonged bleeding, less and larger platelets, reduced platelet \(\alpha\)-granule content and a reduction in killing capacity of cytotoxic T cells [103].

4.8. \textit{Mevalonate kinase deficiency} (MIM 251170, 260920)

MK deficiency is the only disorder, which affects the biosynthesis of all cellular isoprenoids. It has two phenotypes, mevalonic aciduria (MA) and hyper-IgD and periodic fever syndrome (HIDS). All patients suffer from recurrent fever episodes and generalized inflammation. Therefore, the disease can be considered as an auto-inflammatory disorder. The biochemical and genetic aspects of MK and its deficiency are the scope of this thesis.

5. \textbf{Outline of this thesis}

At the start of this project the cause of HIDS was still elusive. However, the accidental finding of mevalonate in urine of one HIDS patients led to the elucidation of the molecular and biochemical basis of HIDS (chapter 3). The molecular and biochemical basis of MA was already known, but only few disease-causing mutations had been reported. Chapter 4 describes the molecular basis of MK deficiency in three MA patients. In chapter 5 we present the three most common mutations in the gene encoding MK (\textit{MVK}). One of these common mutations is specific for HIDS. The carrier frequency for this mutation has been determined in The Netherlands (chapter 7). Chapter 6 describes the elucidation of the genomic structure of \textit{MVK} and presents an overview of mutations identified in 27 patients with MK deficiency. In chapter 8 and 9 the study of the biochemical consequences of MK deficiency is described. All current knowledge of the biochemical and genetic aspects of MK and its deficiencies is reviewed and discussed in chapter 2.

\textbf{References}

General introduction