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Mevalonate Kinase Deficiency: identification of new therapeutic target, *in vitro* and *in vivo* pathogenic study

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RIASSUNTO

Il Difetto di Mevalonato Chinasi (MKD) è una malattia rara autoinfiammatoria autosomica recessiva, causata da mutazioni nel gene *MVK* che codifica per mevalonato chinasi (MK), enzima chiave della via del mevalonato. Questa via è importante per la produzione di colesterolo, ed anche geranilgeranil pirofosfato e farnesil pirofosfato essenziali per la prenilazione delle proteine. MKD ha fenotipi clinici eterogenei, infatti, si va da una forma lieve, la sindrome iper-IgD (HIDS), ad una forma più grave, la Mevalonica Aciduria (MA). HIDS è caratterizzata da sintomi eterogenei che comprendono febbri ricorrenti, eruzioni cutanee, afte, artralgia, dolori addominali, diarrea e vomito; mentre MA oltre a tutto questo, mostra un fenotipo più grave con coinvolgimento neurologico, ritardo psicomotorio, epatopatia e atassia cerebellare. Più del 50% dei pazienti MA muore durante l'infanzia o nella prima infanzia.

Non è tuttora chiara la correlazione tra mutazioni di *MVK* ed il fenotipo clinico di MKD; infatti a causa della grande eterogeneità genetica e clinica, la correlazione genotipo-fenotipo risulta essere problematica.

L'MKD ad oggi è ancora una malattia orfana di trattamento eziologico specifico, sono ancora poco conosciuti i meccanismi patogenetici ed i principali attori coinvolti nella malattia; in particolar modo, non è ancora stata chiarita la patogenesi collegata alle gravi manifestazioni cliniche di MA così come i meccanismi neuro-infiammatori e le interazioni che avvengono tra i diversi tipi cellulari nel sistema nervoso centrale.

L'ipotesi patogenetica di MKD ad oggi più accreditata collega il fenotipo infiammatorio con la diminuzione di composti isoprenoidi e del livello di proteine prenilate, causato dal blocco della via del mevalonato. Questa diminuzione di proteine determina attivazione dell'inflammosoma NALP-3, che a sua volta induce l'attivazione ed rilascio di IL-1β.

Attualmente, vi è una mancanza di modelli per lo studio dell'MKD. Infatti, il modello biochimico ottenuto *in vivo e in vitro* mediante somministrazione di inibitori della via del mevalonato (aminobifosfonati o statine) è l'unico modello in grado di mimare le caratteristiche patologiche.

L'obiettivo di questo progetto di dottorato è indagare il meccanismo patogenetico del Difetto di Mevalonato Chinasi, ponendo particolare attenzione alla forma più grave, la Mevalonico Aciduria, valutando i meccanismi neuro-apoptotici e neuroinfiammatori tipici di questa sindrome.

Per tutti questi motivi, abbiamo eseguito l'analisi dell'esoma di pazienti MKD, per valutare la presenza di eventuali altri geni implicati nelle variazioni fenotipiche; studiato, in modelli biochimici *in vitro* (ottenuti in cellule neuronali, microgliali e monocitiche), i meccanismi patogenetici di MKD, tra cui l'apoptosi, il danno mitocondriale, lo stress ossidativo e l'infiammazione; abbiamo inoltre valutato l'infiammazione sistemica e la neuro-infiammazione nel modello biochimico *in vivo,* ottenuto in due diversi ceppi di topi (BALB/C e C57BL/6); infine, abbiamo sviluppato un modello genetico *in vitro,* utilizzando trasfezione transitoria di due differenti mutazioni tipiche di MKD (I268T associato ad HIDS, e N301T tipico di MA), valutando le basi molecolari della malattia e il meccanismo patologico legato al processo autofagico.

I principali risultati che sono emersi da questo lavoro di tesi sono:

- *GRID2* potrebbe essere un gene modificatore di MKD;
- il blocco biochimico della via del mevalonato nelle cellule neuronali causa un equilibrio tra apoptosi, che segue la via mitocondriale (caspasi-9 e caspasi-3 dipendente), e piroptosi (caspasi-1 dipendente);
- il blocco biochimico induce attivazione della microglia che a sua volte determina un ulteriore incremento del livello di apoptosi nelle cellule neuronali;
- nel modello biochimico *in vivo,* ottenuto in due diversi ceppi di topi, si registra un'infiammazione sistemica e neuronale;
- il blocco della via del mevalonato induce danno mitocondriale, stress ossidativo e rilascio di citochine pro-infiammatoria che portano le cellule verso l'apoptosi finale;
- nel modello genetico *in vitro* di MKD ottenuto in cellule neuronali, le mutazioni di *MVK* causano apoptosi collegata ad alterazione del flusso autofagico.

I risultati ottenuti durante questo progetto di dottorato ci hanno permesso di formulare una nuova ipotesi patogenetica di MKD basata sul difetto della mitofagia, un meccanismo in cui il danno mitocondriale è collegato al difetto dell'autofagia.

ABSTRACT

Mevalonate Kinase Deficiency (MKD) is a rare autoinflammatory autosomal recessive inborn disease, caused by mutations in *MVK* gene that encodes for Mevalonate Kinase (MK) an important enzyme of the mevalonate pathway. Mevalonate pathway is important for the production of cholesterol, geranylgeranyl pyrophosphate and farnesyl pyrophosphate essential for protein prenylation. MKD has heterogeneous clinical phenotypes, with a mild form, Hyper-IgD Syndrome (HIDS), and a severe one, Mevalonic Aciduria (MA). Heterogeneous symptoms including recurrent fevers, cutaneous rash, aphtae, arthralgia, abdominal pain with diarrhoea and vomiting characterize HIDS, while MA shows a more critical neurologic phenotype with psychomotor retardation, hepatopathy and cerebellar ataxia. More than 50% of MA patients die in infancy or early childhood.

The correlation between *MVK* mutations and MKD clinical phenotype is still to be elucidated. Genotype-phenotype correlation is sometimes problematic due to the great genetic and clinical heterogeneity. MKD is also an orphan drug disease and the pathogenic mechanisms as well as the main actors involved in disease's aetiology are still unknown; especially the pathogenesis of MA clinical manifestations has not been established. Indeed, the neuro-inflammatory mechanisms and the interactions that occur between the different cellular types in the brain have not yet been explained.

The most accredited MKD pathogenetic hypothesis is based on the evidence that the mevalonate pathway block induces a decrease in isoprenoid compounds and prenylated proteins, leading to inflammatory phenotypes, caused by the activation of NALP-3 inflammasome that consequently determines IL-1β activation.

Currently there is a lack of models for MKD studies. Indeed, the only model able to mimic pathologic features is a biochemical model obtained *in vivo* and *in vitro* by administration of mevalonate pathway inhibitors such as aminobisphosphonate or statin.

The aim of this PhD project is to investigate the pathogenic mechanism of MKD. Special attention is given to MA, in order to evaluate the neuro-apoptotic and neuro-inflammatoy mechanisms leading to this syndrome.

For all these reasons, we performed exome analyse of MKD patients in order to evaluate the presence of eventual other modifiers gene, able to modulate MKD phenotype; we investigated pathogenic mechanisms of MKD, including apoptosis, mitochondrial damage, oxidative stress and inflammation using an *in vitro* biochemical models (i.e., neuronal, microglia and monocytic cells); we also evaluated systemic inflammation and neuro-inflammation employing an *in vivo* biochemical model obtained in two different mice strains (BALB/c and C57BL/6); finally, we developed an *in vitro* genetic model using transient transfection of two different MKD mutations (I268T associated with HIDS, and N301T typical of MA), evaluating the molecular basis of MKD and the pathology mechanism linked to autophagy.

The main specific results emerging from this PhD thesis work are:

- *GRID2* could be a modifier gene of MKD;
- biochemical block of mevalonate pathway in neuronal cells caused a balance between apoptosis follows mitochondrial pathway (caspase-9 and caspase-3 dependent) and pyroptosis (caspase-1 dependent);
- microglial activation is a direct consequence of mevalonate pathway block, which induces an additional increase of neuronal cell death;
- systemic and neuronal inflammations are observed in biochemical *in vivo* model obtained in two different mice strains;
- mevalonate pathway block induced mitochondrial damage, leading to oxidative stress and pro-inflammatory cytokines' release, which leaded cells to final apoptosis;
- *MVK* mutations cause an alteration in autophagic flux that leads cells to final apoptosis, in *in vitro* genetic model of MKD in neuronal cells.

The findings obtained during the PhD enabled to formulate a new MKD pathogenic hypothesis, based on mitophagy impairment, a convergent mechanism in which mitochondria damage plays a pivotal role contributing to apoptosis and autophagy impairment.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Mevalonate Kinase Deficiency

Mevalonate Kinase Deficiency (MKD) is a pediatric disease originally identified by van der Meer JW. et al. in 1984 [1]; nowadays, is considered a member of hereditary periodic fever syndromes (HPFSs).

HPFSs are rare, distinct, heritable disorders characterized by intermittent and recurrent attacks of fever and localized inflammation. During these attacks, systemic inflammation, characterized by elevated serum levels of acute-phase reactants (e.g., fibrinogen, serum amyloid A), leucocytosis and an elevated erythrocyte sedimentation rate are present. Between attacks, patients are non-symptomatic [2].

In the last few years, six HPFSs have been well identified (see Figure 1).

Syndrome	Gene and Locus	Protein	Mode of Inheritance
FMF	*MEFV*, 16P13.3	Pyrin, marenostrin	Autosomal recessive
HIDS	*MVK*, 12q24	Mevalonate kinase (MK)	Autosomal recessive
TRAPS	*TNFRSF1*, 12p13	TNF-receptor type 1	Autosomal dominant
MWS	*NLRP3 (CIAS1)*, 1q44	Cryopyrin (NALP3/ PYPAF1)	Autosomal dominant
FCAS	*NLRP3 (CIAS1)*, 1q44	Cryopyrin (NALP3/ PYPAF1)	Autosomal dominant
CINCA	*NLRP3 (CIAS1)*, 1q44	Cryopyrin (NALP3/ PYPAF1)	Autosomal dominant

Figure 1: Six member of hereditary periodic fever syndrome identified: gene symbols, chromosomal loci, protein products, and modes of inheritance of diseases [3].

MKD is a rare, autosomic recessive, metabolic disease linked to homozygous or compound heterozygous mutations in the *MVK* gene (12p24,11; *MVK*), coding for the enzyme mevalonate kinase (ATP:mevalonate 5-phosphotransferase; EC 2.7.1.36; MK) on the cholesterol pathway [4].

The catalytic activity of MK appears to be inversely related to the severity of the disease. Indeed, in the mild form, Hyper-IgD Syndrome (OMIM#260920; HIDS), enzymatic activity is between 1% and 8%, instead, in the severe form, Mevalonic Aciduria (OMIM #610377; MA) MK activity is below the lower levels of detection (less than 1%) [5].

The study conducted by Simone et al., allowed establishing that HIDS and MA were not two distinct diseases: by means of patients' clinical observation, the authors suggested that both diseases are a continuous spectrum of MKD with increasing severity [6].

HIDS patients are suffering from recurrent fever attacks (>38.5 °C), with skin rashes, hepatosplenomegalia and generally a sustained inflammatory response, whereas, the more severe form MA, is characterized by the involvement of the Central Nervous System (CNS), with cerebellar ataxia, psychomotor retardation and also, as in HIDS, recurrent fever attacks [7,8]. During these fever attacks it occurs a production of pro-inflammatory cytokines interleukin-1α (IL-1α), IL-1β, IL-6, interferon-γ (IFN-γ) and tumor necrosis factor α (TNFα) [9,10].

Usually, the first attack begins before the end of the first year of life. The attacks, which are typical in late infancy and childhood, tend to diminish with increasing age, although symptoms usually continue throughout life. Some HIDS patients even attain spontaneous complete disease remission [8].

The MA shows great clinical heterogeneity being generally more severe than HIDS, in fact in MA more than 50% of patients die in infancy or early childhood [11]. Patients' long-term outcome survived the early childhood is poorly understood, and there is no information on adolescent patients [12].

The pathogenesis of the clinical manifestation of Mevalonic Aciduria has not been yet established. Case report shows MA patients with progressive atrophy of the cerebellum, through neuroimaging studies. In particular, this cerebellar atrophy is characterized by a selective and progressive decrease of anatomical mass of the cerebellum, and appears to be proportional and direct consequence of progressive neuronal death. This clinical observation reflects a gradual increase of psychomotor retardation in patients [13].

However, the neuro-inflammatory mechanisms and the interactions that occur between the different cellular types in the brain have not yet been explained.

1.2 Diagnosis of Mevalonate Kinase Deficiency

MKD diagnosis is often difficult since awareness for these rare and relatively new disease is still low for general practitioners. Furthermore, MKD comes with several, and not always present, typical clinical features, such as high levels of IgD, IgA, diarrhea, vomiting, abdominal pain. During the inflammatory attacks there are always high levels of Erythrocyte sedimentation rate, C-reactive protein, or Serum Amyloid A protein [14,15].

In order to diagnose Mevalonic Aciduria, is important to detect the high titer of mevalonic acid in urine, cerebrospinal fluid and plasma. Indeed, in HIDS patients too during febrile attacks, it is possible to register significant increases of mevalonic acid.

However, MKD seems very unlikely in patients with normal mevalonic acid excretion, but it cannot be excluded completely, in the same way, a positive urinary mevalonic acid excretion cannot be related only to MKD [16]. For all these reason the diagnostic evaluations should be confirmed by biochemical and genetic analysis, designed respectively to measure the enzyme activity (MK activity in leukocytes or cultured skin fibroblasts) and to identify possible mutations of *MVK* gene [17]. Infevers, a mutations database for periodic fevers, reports and widely describes all published *MVK* mutations [18].

In 2015, Federici S at al. have validated a set of clinical criteria for the classification of patients affected by periodic fever, called Eurofever Classification Criteria. These criteria give a high sensitivity score based on clinical variables that were independently correlated to each disease. In this manner, the diagnosis is facilitated especially in the presence of unpublished genetic mutations or when the genetic testing is not clearly confirmatory [19].

Despite the various studies conducted recently, the MKD diagnosis is still very difficult, especially when concerning the distinction between mild MA and HIDS, due to clinical manifestations overlapping and to poor genotype–phenotype correlation [20,21].

Nowadays, prenatal diagnosis is possible with mevalonate measurement, enzyme activity evaluation (in cultured amniocytes and biopsied chorionic villus) and mutations study [22,23]

The international register of orphan diseases, Orphanet, updated to 2006, counts 180 HIDS patients and 30 MA patients worldwide [24]. Nevertheless, the diagnostic difficulties suggest that these numbers are an underestimate of the true incidence of the MKD disease.

1.3 Therapeutic interventions in Mevalonate Kinase Deficiency

Currently, MKD is an orphan drug disease. The adopted therapy is based on an empirical method and the most used drugs are: colchicine, cyclosporine, thalidomide, paracetamol during febrile attack; prednisolone and corticosteroids in the early phase of attacks, aimed at reducing its severity and duration; non-steroidal anti-inflammatory drugs during inflammatory attacks [11,15,17,22,25,26].

Over the years, compounds able to act on the cholesterol pathway, such as statins that decreased attacks frequency has been used in MKD treatment [27]. Statins are a class of inhibitor of hydroxymethylglutaryl coenzyme A reductase (HMGR), the enzyme that

catalyzes the formation of mevalonic acid. However, in the majority of studies, patients treated with statins did not respond to this therapy [15,21]; moreover, in patients with severe HIDS and MA, statins treatment has been reported as triggering inflammatory attacks [12].

Recently, biological drugs able to oppose the action of pro-inflammatory cytokines have been employed. Among these drugs, the most used are: Infliximab, Abalimumab and Etanercept, tumor necrosis factor α (TNF-α) antagonists; Canakinumab and Rilonacept, IL-1 antagonists; and Anakinra, IL-1 receptor antagonist. Despite the great expectations, in the literature many cases of failure of these drugs are reported [15,28,29]. Among the aforementioned drugs, Anakinra and Etanercept are the most effective ones in MKD treatment. Indeed they reduced the frequency and the intensity of inflammatory and fever attacks. Many studies observed that patients who do not respond to Etanercept treatment could benefit from Anakinra treatment and vice versa [15,30].

In MA patients the replacement of the hematopoietic system with allogeneic bone marrow transplantation from a healthy donor has improved symptoms of inflammation. On the other hand, the long-term effects of bone marrow transplantation on cells and tissues outside the hematopoietic system are still unknown. Currently, this procedure is utilized only in patients with MA whose condition is resistant to biological drugs therapy [31,32]. Moreover, there is an unknown variability in drugs response; therefore there is no single therapy effective in all patients. It is the responsibility of the physician to find the personalized treatment of each patient considering balance between benefits versus risks and costs [15].

Besides the unclear pathogenesis of MKD, it should be kept in mind that this is an orphan drug disease; all therapies given to patients are non-specific and act, with high variability and in a non-reliable manner, only on inflammatory symptoms.

1.4 *MVK* gene

The *MVK* gene is 23576 bp long, consisting of 11 exons and 10 introns and maps on the long arm of chromosome 12 (12q24.11 NM_000431) (Figure 2).

To date, 204 genetic variants have been reported and are consultable in the dedicated Infevers database [18]. Most of the mutations consist of a single nucleotide substitutions or deletions, while a lesser percentage of insertions and duplications.

Even if several *MVK* genetic variants have been described, the *MVK* genotype/MKD phenotype correlation is poor. The identification of *MVK* mutations is only supportive of the diagnosis, which still remains clinical driven [33]. Over the years, genetic diagnostic

screenings have been proposed to distinguish the mild form HISD from severe MA, based on *MVK* genetic variations, but the results have been quite poor [34].

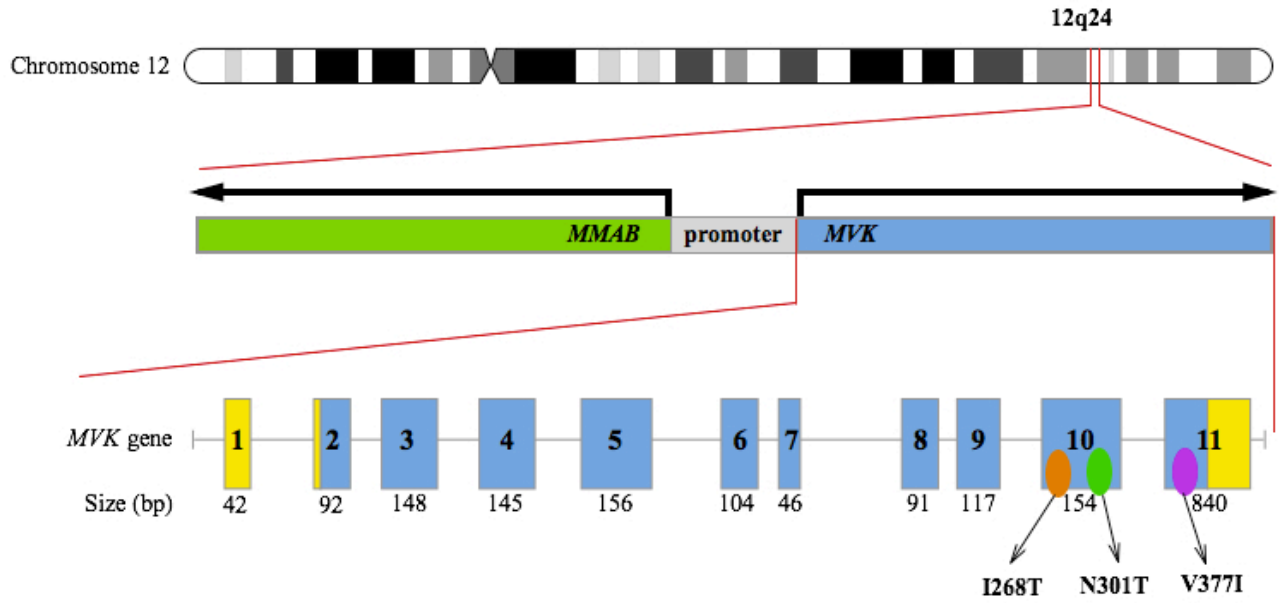

Figure 2: Representation of chromosome 12 with magnification of genetic region of *MMAB* and *MVK* genes with their shared promoter region. Higher magnification of *MVK* gene with schematic representation of 3 important mutations: I268T, N301Tand V377I.

The most frequent mutation, found in approximately 80% of HIDS is c.1129G>A (rs28934897), present in homozygosis but mostly in heterozygosis, resulting in an amino acid change from valine to isoleucine at position 377 (p.V377I); strikingly, this mutation has not been observed in MA patients [34].

The second most frequent mutation associated to HIDS is c.803T>C (rs104895304). This mutation results in amino acid substitution from isoleucine to threonine at position 268 (p. I268T) and is shared by both HIDS and MA diseases. Indeed, in most HIDS patients we found I268T in association with V377I; moreover, in MA I268T was found associated with another mutation c.902A>C (rs28934896) p. N301T [34]. However, these associations are not always existent and patients with the same mutation often present different clinical phenotypes, so the genotype-phenotype correlation is in some cases very problematic.

The great MKD clinical heterogeneity has also been hypothesized as possibly related to the presence of modifier genes or related to the presence of other typical mutations of autoinflammatory disorders. For these reasons, in recent years studies have been performed also using next-generation sequencing; unfortunately, to date there are no significant results, which enable to better understand the genetics of MKD [33,35].

Then it has been also thought to evaluate the *MVK* promoter action that it is shared with an

adjacent gene, MMAB, coding for Cob(I)alamin adenosyltransferase, an enzyme converting cob(I)alamin to adenosylcobalamin, an important co-factor in mitochondrial methylmalonyl-CoA mutase (Figure 2). The two genes are in head-to-head conformation; it has also been shown that lower cholesterol levels induce increase of MVK mRNA and protein and of MMAB mRNA [36]. However, this interesting study is still in progress.

1.5 MK enzyme

MK (ATP (R) -mevalonato 5-phosphotransferase, NM_000431.2) is an enzyme of 396aa, localized in the cytosol and peroxisomes, member of the GHMP kinase family [37,38]. MK is an important enzyme in the biosynthesis of cholesterol, also known as "mevalonate pathway". MK catalyzes the transfer of the γ-phosphoryl group from ATP to the C5 hydroxyl oxygen of mevalonic acid to form mevalonate 5-phosphate. MK is an homodimeric enzyme and each subunit is characterised by four domains: a peroxisomes targeting sequence 2 (PTS2) domain in N-terminal portion, binding site for ATP in C-terminal portion, and other two domains between them. The active site is located in a cleft at the domain interface and in particular there are four amino acids that play a crucial role in the enzymatic activity (K13, E19, E193 and D204) [39,40] (Figure 3).

It is not known how MKD mutations are able to modify the enzyme structure or how this contributes to the different forms of disease's severity. Recently, an interesting article reported the prediction of the effects of *MVK* known mutations in the structure of MK enzyme, identifying two hot spot regions (comprised between residues 8-35 and 234-338) for MA. These two regions are located around the inside of the protein's cleft close to the active site and around the domain interface [41] (Figure 4). Therefore, the variation of these amino acid residues could be at the basis of low MK activity found in MA.

The catalytic activity of MK is inversely related to the severity of the disease. Indeed, in HIDS the enzymatic activity was from 1% to 8%, while in MA the MK activity was below the levels of detection (less than 1%). Therefore, MA mutations could disrupt the enzyme's structure and function altering protein folding and stability.

Additionally, MK enzymatic activity seems to be influenced by temperature. Houten SM. and collaborators observed that febrile temperatures induced a further decrease of MK activity and a successive worsening of inflammatory phenotype [42].

V377I, the most frequent mutation associated to HIDS, seems not to affect the catalytic activity but rather alters the maturation of the mutant protein into an active enzyme. This

mutation encodes a polypeptide that apparently is not capable of folding efficiently in the correct physiological conformation [42,43].

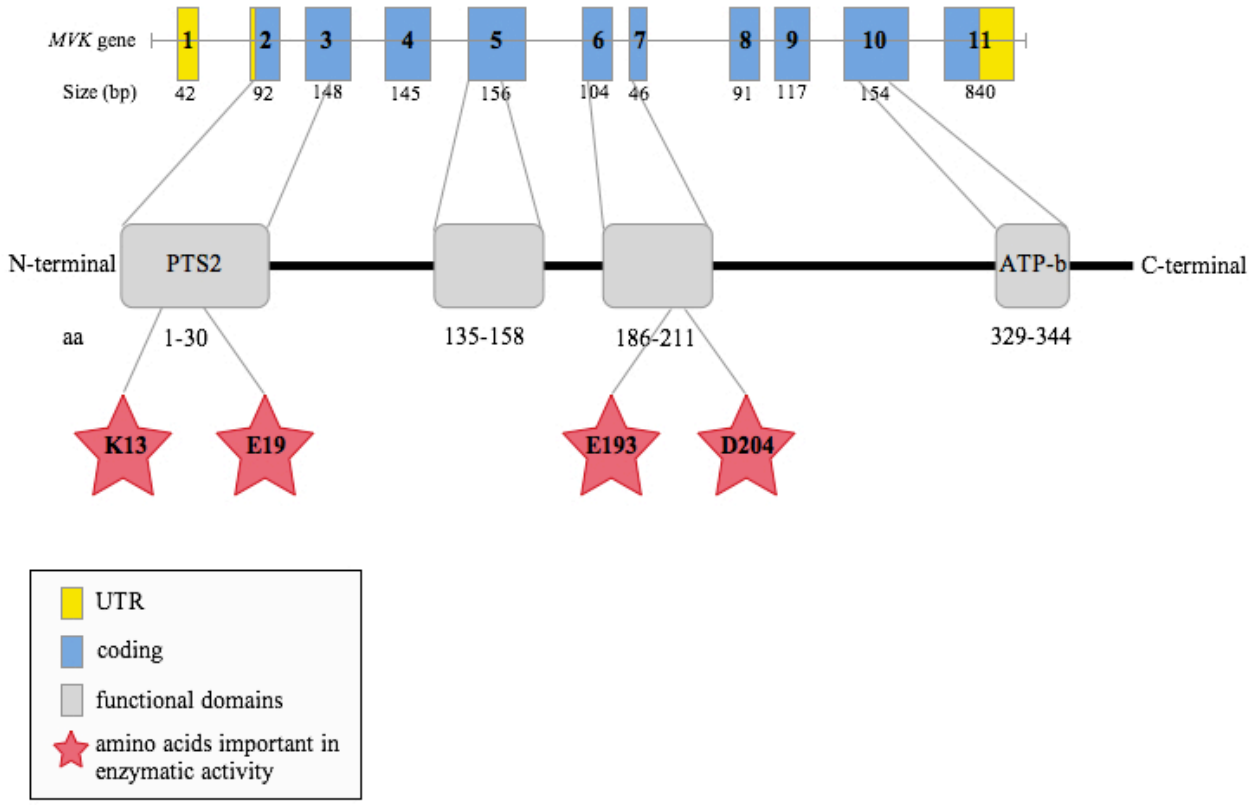

Figure 3: Genomic structure of MVK gene and the predicted functional four domains of mevalonate kinase proteins: N-terminal-peroxisome targeting signal 2 (PTS2), C-terminal ATP binding (ATP-b), and other two domains between them. Magnification of four amino acids that play a crucial role in the enzymatic activity (K13, E19, E193 and D204).

I268T, the second most frequent mutation in HIDS, occurs in a region important for dimerization of the protein. This amino acid changes may weaken the dimerization interaction, making the protein somewhat less stable [40].

These bioinformatics approaches are useful to better understand the structural and functional consequences of *MVK* mutation in order to better achieve the genotype-phenotype correlation.

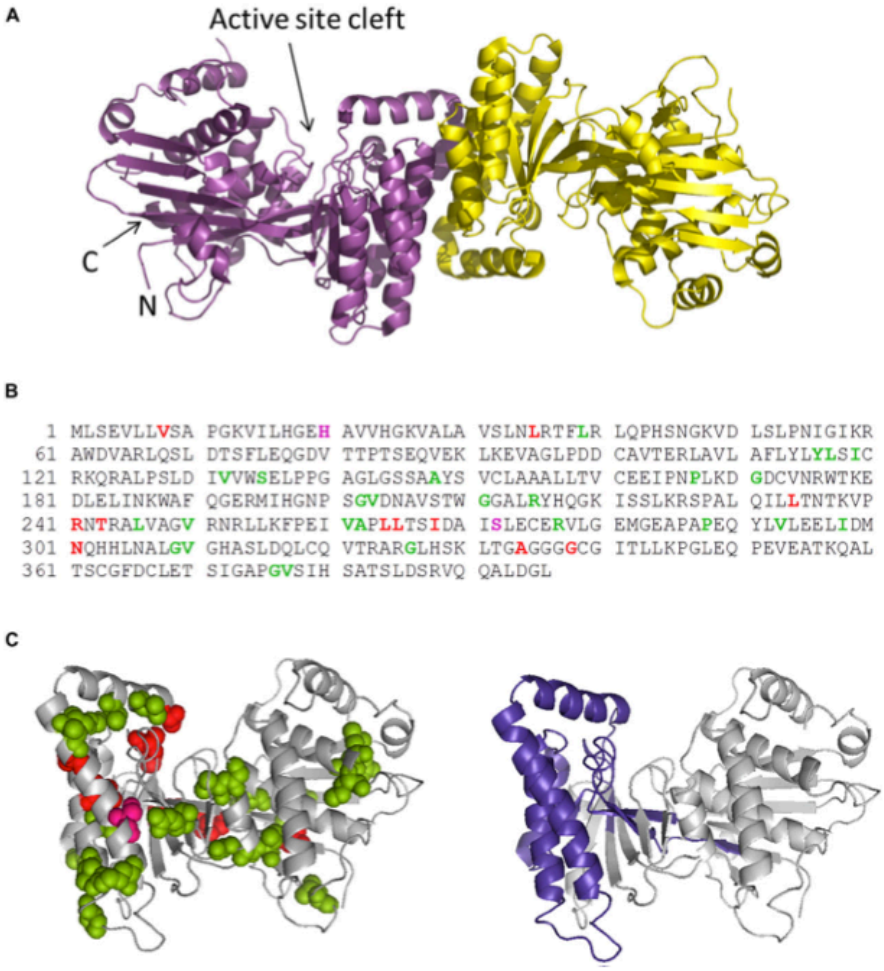

Figure 4: The dimeric structure of mevalonate kinase protein (MK) showing the N- and C-termini and active site cleft of one of the subunits. (B) The sequence of human MK showing tipical residues altered in HIDS (green), intermediate (magenta), and MA (red) . (C) These residues mapped onto the three-dimensional structure of a single MK subunit (left). tipical residues altered in HIDS (green), intermediate (magenta), and MA (red). The hot spot regions (comprised in residues 8-35 and 234-338) which includes all the alterations associated with MA is coloured blue in the right-hand figure [41].

1.6 Mevalonate pathway

Mevalonate pathway is important for the production of key intermediate of cholesterol and non-cholesterol pathways (Figure 5). Cholesterol pathway determines the production of cholesterol (fundamental constituent of cell membranes), which in turn is converted to steroid hormones, vitamin D and bile acid production.

Instead, non-cholesterol pathway produces dolichols (freeradical scavenger in cell membranes), ubiquinones (electron transporters in mitochondria), geranylgeranyl pyrophosphate and farnesyl pyrophosphate (Figure 5).

Geranylgeranyl pyrophosphate and farnesyl pyrophosphate are regulators of cellular homeostasis key proteins, through a post-translation modification, prenylation, in which a

geranylgeranyl or farnesyl moiety is attached to the protein C-terminus, targeting the modified protein to membranes. Activation of an important class of protein, namely small GTPase of the Ras superfamily, is regulated by this mechanism. Small GTPases regulate a several variety of processes in the cell, including growth, cellular differentiation, cell movement, lipid vesicle transport and autophagic flux.

The most finely regulated molecule in mevalonate pathway is HMG-CoA reductase (HMGR) enzyme, the second enzyme in this pathway [44]. The principal regulatory mechanism involves sterol regulatory elements binding proteins (SREBS), activated by cholesterol and isoprenoids shortage, inducing an increase in HMGR transcription [45,46].

MK is the third enzyme of mevalonate pathway and it is regulated at transcriptional level, in a similar manner of HMGR and by feedback inhibition from geranylgeranyl pyrophosphate and farnesyl pyrophosphate [47,48].

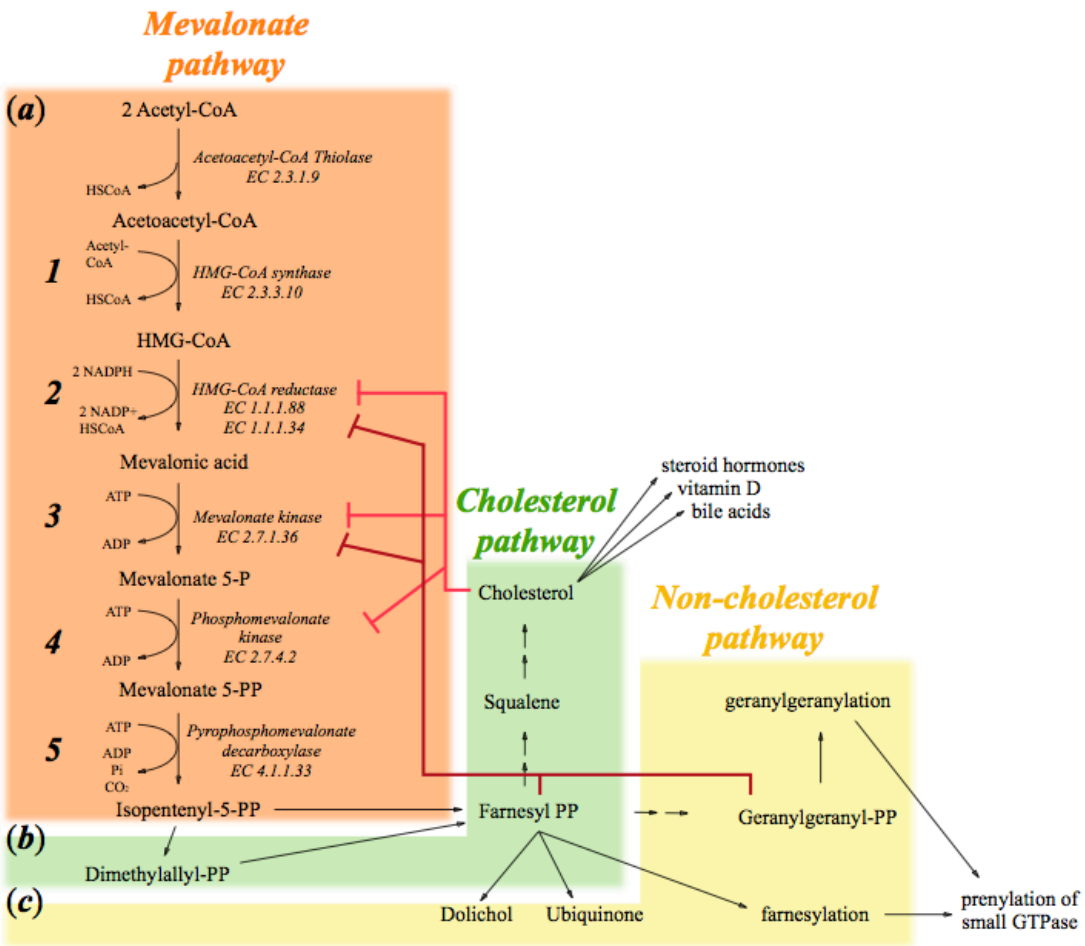

Figure 5: Schematic representation of the mevalonate pathway divided into: (a) the mevalonate pathway (b) the cholesterol pathway and (c) the non-cholesterol pathway [49].

1.7 Pathogenic hypotheses

Presently, the MKD pathological mechanisms are neglected, particularly those concerning the neurological involvement; in fact, the specific molecular events that lead to neurological impairment in MA are still unknown.

Initially, it was thought that the MKD phenotype might be caused by an accumulation of mevalonic acid, the substrate of the deficient MK enzyme. Frenkel J. et al. have discarded this theory and they hypothesized that MKD typical phenotypes could be due to a decrease in mevalonate pathway key products, in particular isoprenoid compounds [50]. To date, this is the most accredited MKD pathogenic hypothesis (Figure 6).

The block of mevalonate pathway is associated to inflammatory process activation also characterized by cytokines production. Most of the cytokines is produced directly in active form; the major exception is interleukin-1β (IL-1β), considered a principal inflammatory marker of MKD [51]. IL-1β is a key mediator of inflammation with a wide range of actions including: synthesis induction of the proteins correlated to acute phase by the liver as the c-reactive protein; the recruitment of neutrophils, monocytes and lymphocytes; fever induction; and also the activation of other important cytokines [52].

It is commonly assumed that IL-1β activation in MKD derives from the activation of the NALP3-inflammasome platform (NACHT, LRR and PYD domains-containing protein 3) a multi-protein complex that recruits pro-caspase-1, which self-cleaves into active caspase-1 and then convert pro-IL-1β and pro-IL-18 to active IL-1β and IL-18, activating one of the main pathway of inflammation [53,54]. In fact, an increase in NALP3 expression was previously reported in studies conducted in peripheral blood mononuclear cells from MKD patients [55].

In MKD patients, activation of NALP3 inflammasome with consequent release of IL-1β may cause fever attacks [56]. Instead, at cellular level, activation of NALP3 inflammasome may induce an increase of caspase-1 dependent programmed cell death, also known as pyroptosis [57].

It has been recently demonstrated that natural isoprenoids, such as geraniol, farnesol, geranylgeraniol, and menthol, are able to rescue the MKD-inflammatory phenotype, by affecting the production of cytokines (IL-1β, IL-18, TNF-α) and preventing cell death in both *in vitro* and *in vivo* studies [58]. These results confirm the most widely accepted MKD pathogenic hypothesis.

Mevalonate pathway block may be correlated to oxidative stress and mitochondrial damage

[59]. Reactive Oxygen Species (ROS) are produced when mitochondrial damage and oxidative stress occur: they play a role in activating the inflammasome molecular platform, which in turn induces pro-inflammatory cytokines release [60,61]. Mevalonate pathway produces important metabolites with antioxidant activity, such as ubiquinone, dolichol, and heme A and blockade of this pathway, as in MKD, induces shortage of these metabolites [62,63]. All these aspects are additional confirmations of MKD pathogenic hypothesis.

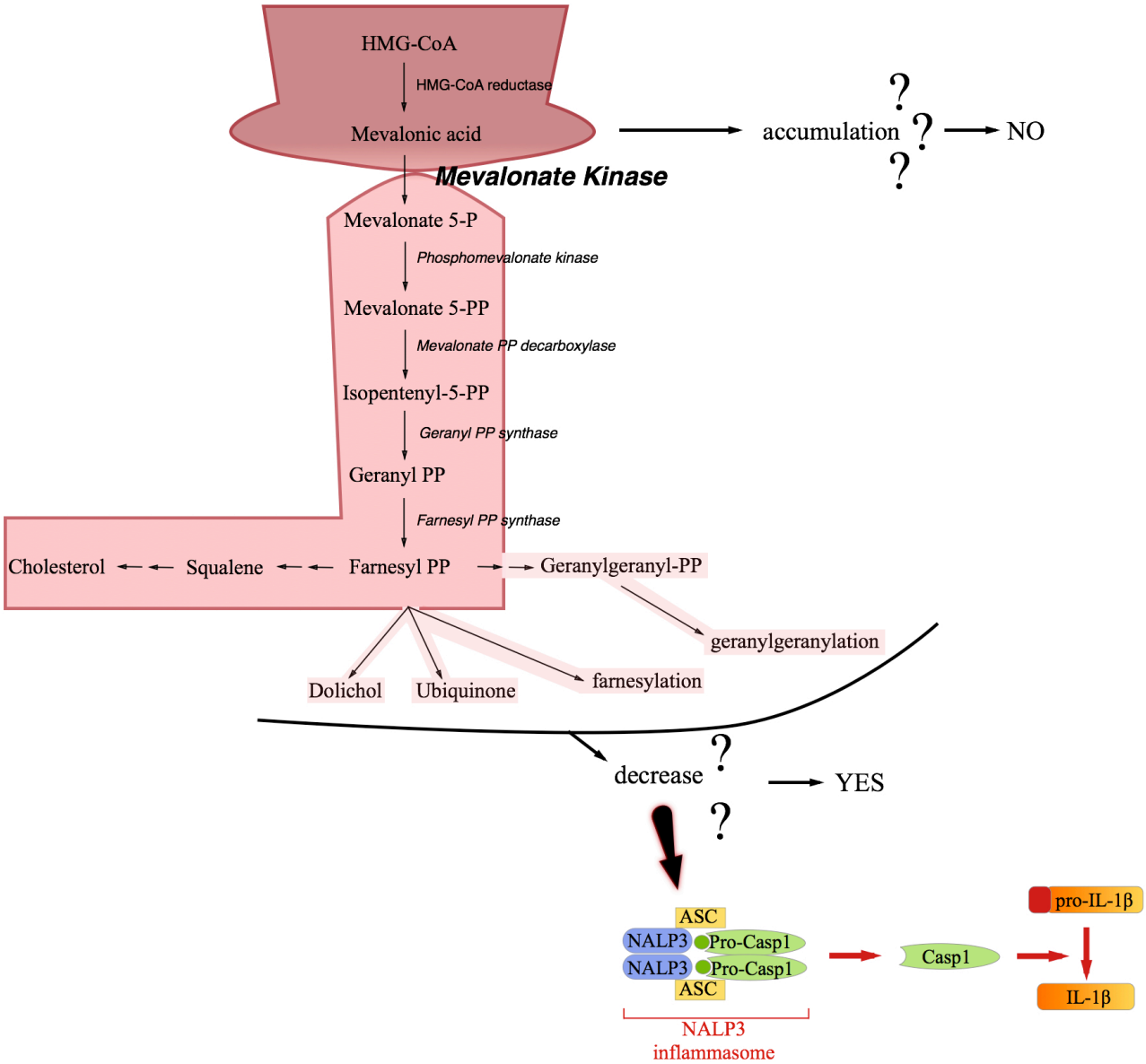

Figure 6: Schematic representation of mevalonate pathway and MKD pathogenic hypothesis. First hypothesis, based on the accumulation of mevalonate, was discarded. The second hypothesis is the most accredited one: *MVK* mutation induces mevalonate pathway blockage subsequently causing decrease of isoprenoid compounds and end products of the mevalonate pathway. All these events are important for the activation of NALP3 inflammasome that cleaves and activates IL-1β.

Nevertheless, the precise pathogenic mechanism is still unclear, but his understanding is

essential to design a possible specific pharmacological treatment.

1.8 MKD models

MKD models able to mimic principal disease characteristics are important to understand its pathogenic mechanisms and to test possible new drugs.

To date, the only existing genetic model of disease is an *in vivo* MKD model, obtained in mice by a deletion of a single allele of the gene *MVK*, *MVK* mice (+/-). Instead, a complete loss of gene function, as in mouse homozygous *MVK* (-/-), appears to be lethal. In blood and tissues of mice *MVK* (+/-), normal levels of cholesterol and isoprenoids, and significant increases serum levels of IgA and IgD were measured. The main limitation of this *in vivo* genetic model is that the residual enzymatic activity is around 50%, and the absence of neurological dysfunction [64]. Other similar model are the deletion of specific genes in cholesterol pathway which caused negative outcomes in mice; indeed, ablation of *HMG-CoA* reductase gene, *HMGCR* (-/-), and squalene synthase gene, *SQS* (-/-), results in embryonic lethality in mice [65,66].

In 2012 Wang P et al. presented another *in vivo* MKD genetic model: they generated a mouse model that stably and efficiently expresses secretory IgD. Nevertheless, this model did not show typical MKD phenotypic characteristics [67].

Subsequently, our research group tried to develop a novel cellular MKD model in which MVK expression is specifically down regulated, using silencing RNA (siRNA) technology: MK protein reduction of up to 40% has been obtained in this model, but, this reduction has not been sufficient to cause inflammasome activation and cell death. So, this model was not able to mimic the pathologic features [68].

However, a biochemical MKD model able to reproduce the genetic deregulation on the mevalonate pathway has been developed. Mevalonate pathway blockade was obtained using compounds, such as aminobisphosphonates and statins, known to inhibit different enzymes of this biochemical pathway. Aminobisphophonate inhibits farnesyl pyrophosphate synthase (FPPS), an enzyme of the mevalonate pathway downstream MK. Instead, statin inhibits HMG- CoA reductase, an enzyme of the mevalonate pathway upstream MK. These inhibitors induced the shortage of isoprenoid compounds as well as final products of the metabolic cholesterol pathway [69,70] (Figure 7). In these models bacterial compounds are used to trigger strong inflammatory attacks, such as muramyldipeptide (MDP) or lipopolysaccharide (LPS), similarly to what described in patients with MKD, during febrile and inflammatory

attacks.

The *in vivo* biochemical model, obtained in BALB/c, is characterized by significant increase of inflammatory markers such as serum amyloid A and a number of cells in the peritoneal exudate [71]. During acute inflammatory reaction there are a rise in body temperature accompanied by a significant increase of some cytokines, which appear to have a similar deregulation in MKD patients [72,73].

Since the biochemical model follows current pathogenic hypothesis inducing decrease of isoprenoid compounds and products of the mevalonate pathway, it was used for better disclosing MKD pathogenesis and testing potential MKD drugs, in several studies *in vivo* and *in vitro*.

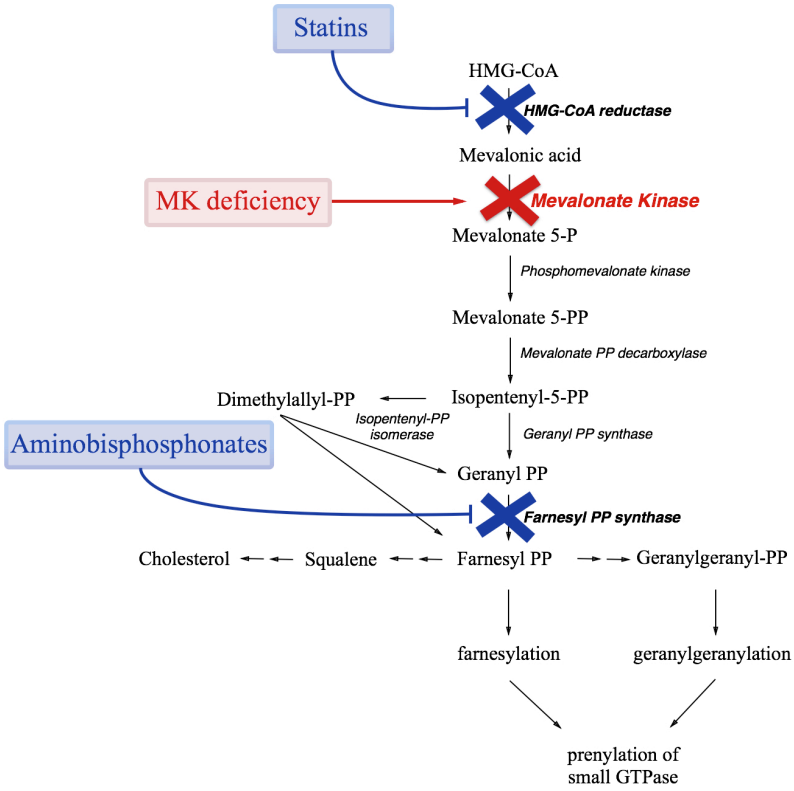

Figure 7: Schematic representation of the mevalonate pathway. Mutation in *MVK* that encodes for MK (red) induces MKD. Alongside the pathway are indicated compound used to reproduces biochemical MKD model: statin that inhibits HMG- CoA reductase, and aminobisphosphonate inhibits farnesyl pyrophosphate synthase (FPPS).

1.9 Aim

The aim of this PhD project is to investigate the pathogenic mechanism of Mevalonate Kinase Deficiency (MKD). Special attention is given to Mevalonic Aciduria, in order to evaluate the neuro-apoptotic and neuro-inflammatoy mechanisms leading to the more severe form of MKD.

The study of pathogenic mechanism is essential for increasing knowledge on the poor genotype-phenotype correlation in MKD, possibly contributing to develop novel therapeutic strategies.

Therefore, during the PhD the following research activities have been performed:

- to analyse the exome (using an Illumina Exome chip) of MKD patients in order to evaluate the presence of other modifiers gene able to modulate the MKD phenotype (Chapter 2);
- to design and develop *in vitro* biochemical models (i.e., neuronal, microglia and monocytic cells) to investigate pathogenic mechanisms of MKD, including apoptosis, mitochondrial damage, oxidative stress and inflammation (Chapter 3-4-6);
- to study *in vivo* biochemical model in two different mice strains (BALB/c and C57BL/6) evaluating systemic inflammation and neuro-inflammation (Chapter 5);
- to develop an *in vitro* genetic model for better assessing the molecular basis of MKD related to *MVK* mutations (two analysed in this PhD thesis), by using transient transfection of the two different MKD mutations and understanding the pathology mechanism linked to autophagy (Chapter 7).

CHAPTER 2

MVK AND OTHER POSSIBLE MODIFIER GENES INVOLVED IN MKD CLINICAL PHENOTYPES

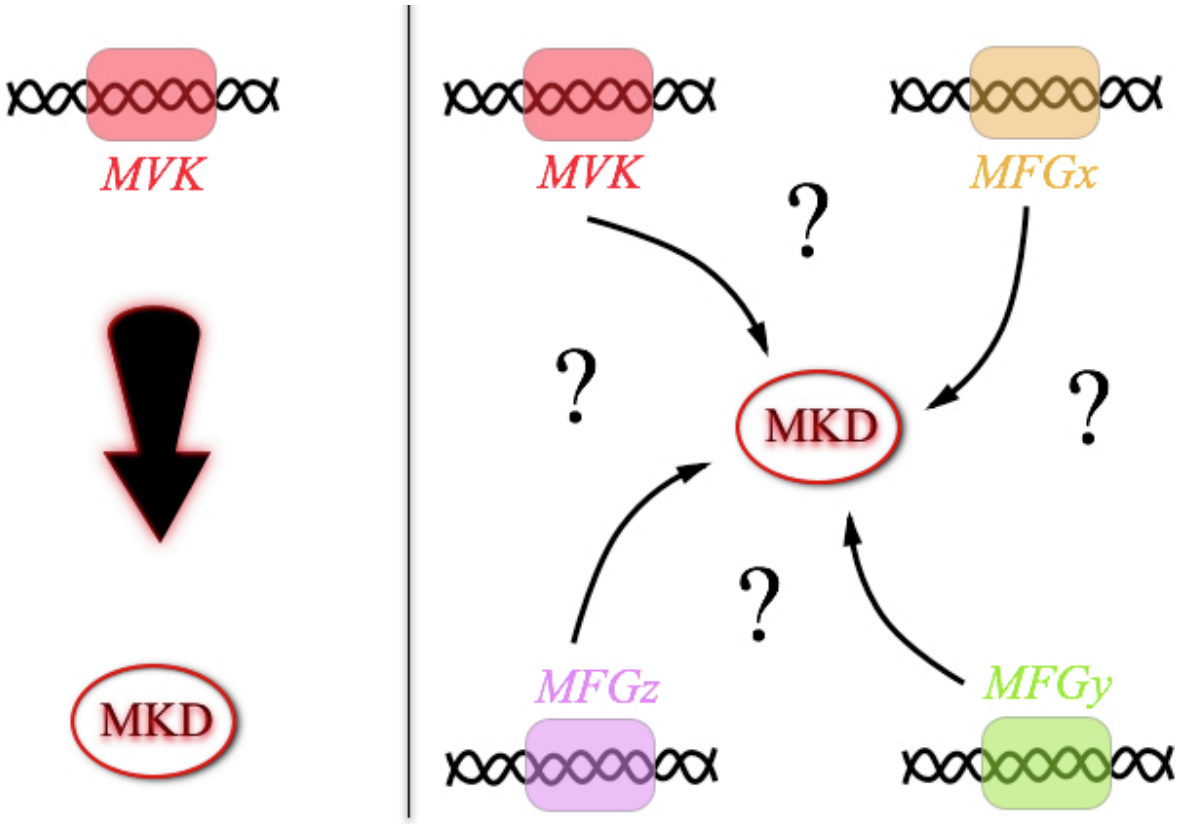

Figure 8: Mutations in *MVK* are responsible for MKD. However, considering the great phenotypic heterogeneity of the diseases we can hypothesize the intervention of other potentially modifier genes (here reported as *MFGx, MFGy, MFGz*) in the modulation of MKD patients' clinical characteristics.

Background: in 1999 Drenth JP and co-workers for the fist time identified several mutations at *MVK* gene in MKD patients [4]. After this observation, MKD was always considered as a Mendelian inherited disease caused by mutations at *MVK* gene. MKD is characterized by poor genotype-phenotype correlation. Indeed, patients carrying the same *MVK* mutations often present heterogeneity of symptoms. This variability causes difficult diagnosis of MKD and inconsistency in responses to therapies. Intervention of other modifiers genes related to patients' genetic background could account for the clinical heterogeneity of MKD patients.

Aim: evaluate the presence of modifier genes other than *MVK* possibly involved in the modulation of MKD phenotype,

Method: exome analysis of MKD patients with Illumina Human Exome bead Chip (release with 240.000 cSNPs and TagSNPs with MAF > 5%).

Principal results: Exome chip findings reveal that GRID2 gene, encoding for human glutamate receptor delta-2, is associated with MKD in a gene-centered analysis; rs1450500 SNP was differently distributed in patients with MKD with respect to those with recurrent fevers.

Conclusions: we hypothesized a possible role for GRID2 as possible phenotype modifier in MKD patients, especially in patients with severe phenotypes (Mevalonic Aciduria).

Ref: "GRID2 a novel gene possibly associated with mevalonate kinase deficiency." Moura R, Tricarico PM, Campos Coelho AV, Crovella S. Rheumatol Int. 2015; 35(4):657-9.

Pubmed link: http://www.ncbi.nlm.nih.gov/pubmed/25146332

CHAPTER 3

MITOCHONDRIAL DAMAGE IN MKD

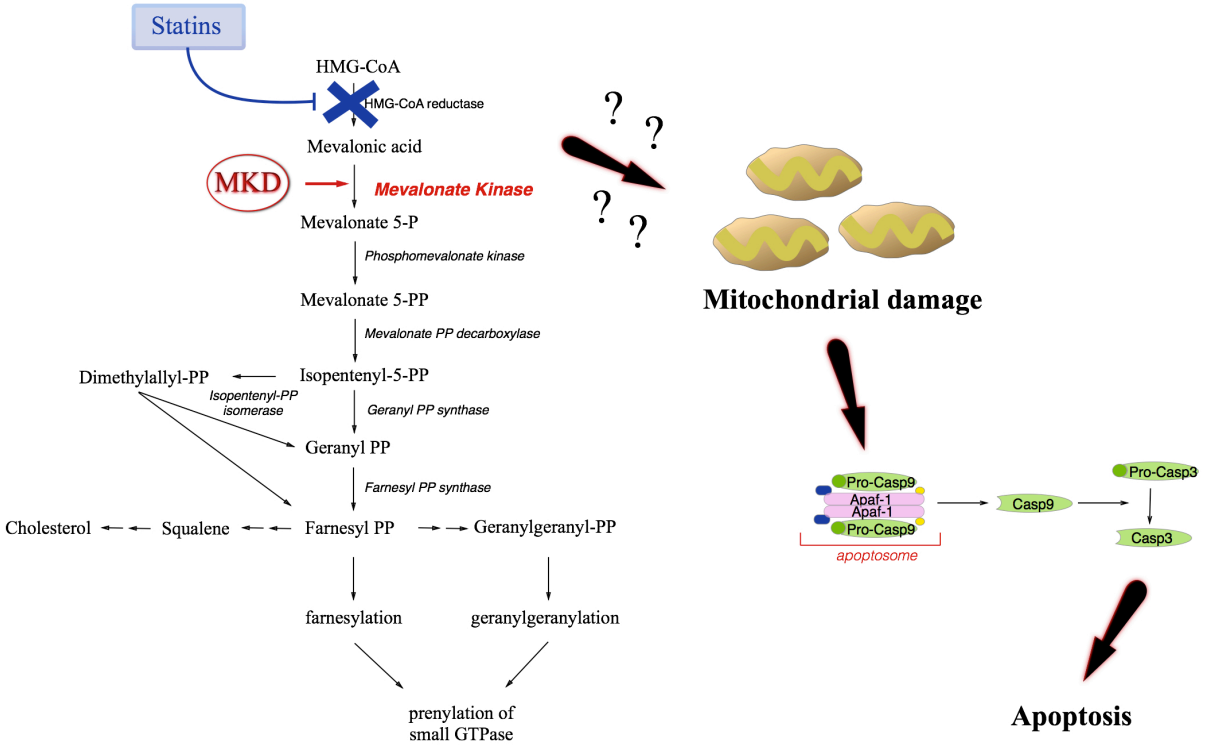

Figure 9: Statins block the mevalonate pathway inhibiting HMG-CoA reductase. Could mevalonate pathway blockage cause mitochondrial damage and subsequent apoptosis through caspase-3 activated by caspase-9 apoptosome?

Background: The most severe form of MKD is Mevalonic Aciduria characterized by recurrent attacks of fever associated with inflammatory symptoms and neurological involvement.

Little is known about the pathogenic mechanisms leading to MA; however, the comprehension of these mechanisms could be very important for the identification of new specific molecular targets for the development of novel therapeutic strategies.

Magnetic resonance imaging of MA patients, revealed severe cerebellum atrophy correlated to a progressive decrease in the cerebellum volume: this progressive decrease could be explained by progressive neuronal cell death [13].

In previous studies we observed an increase of neuronal cell death as a result of mevalonate pathway block, obtained by statin (Lovastatin) administrations in a neuronal cell model (SH-SY5Y): lovastatin blocks mevalonate pathway by inhibiting HMG-CoA reductase (HMGR), mimicking some of the biochemical features found in MKD. Investigating cell death, we demonstrated an apoptotic cell death mediated by the mitochondrial pathway, where caspase-9 is the initiator and caspase-3 the effector caspase [74].

Aim: evaluate the involvement of mitochondria in cell death, by measuring the mitochondrial damage, in biochemical MKD model obtained in neuronal cells (Figure 9). Furthermore, we investigated the intervention of caspase-1, which is important both in inflammatory process that induced maturation of IL-1β, and in caspase-1 dependent programmed cell death, also known as pyroptosis.

Method: we evaluated in neuroblastoma cell line (SH-SY5Y) the programmed cell death and the mitochondrial dysfunction after mevalonate pathway block (obtained by Lovastatin), and after pre-treatment of caspase-3 and/or -9 and/or -1 inhibitors

Principal results: in SH-SY5Y cells Lovastatin induced apoptosis through mitochondrial pathway. Pre-treatment of caspase-3 and -9 inhibitor are not able to completely restore the physiological condition. Indeed, pre-treatment of caspase-1 and -3 inhibitor together and caspase-1 and -9 inhibitors together were able to nullify the Lovastatin- induced effect on mitochondrial damage and programmed cell death.

Conclusions: mevalonate pathway block in neuronal cells induces mitochondrial damage and apoptosis in part sustained by the activation of caspase-3 and in part by caspase-1.

Ref: "Lovastatin induces apoptosis through the mitochondrial pathway in an undifferentiated SH-SY5Y neuroblastoma cell line." Marcuzzi A, Tricarico PM, Piscianz E, Kleiner G, Vecchi Brumatti L, Crovella S. Cell Death Dis. 2013; 4:e585.

Pubmed link: http://www.ncbi.nlm.nih.gov/pubmed/23579272

"Mevalonate kinase deficiency and neuroinflammation: balance between apoptosis and pyroptosis." Tricarico PM, Marcuzzi A, Piscianz E, Monasta L, Crovella S, Kleiner G. Int J Mol Sci. 2013; 14(12):23274-88.
Pubmed link: http://www.ncbi.nlm.nih.gov/pubmed/24287904

CHAPTER 4

MICROGLIAL ACTIVATION IN MKD

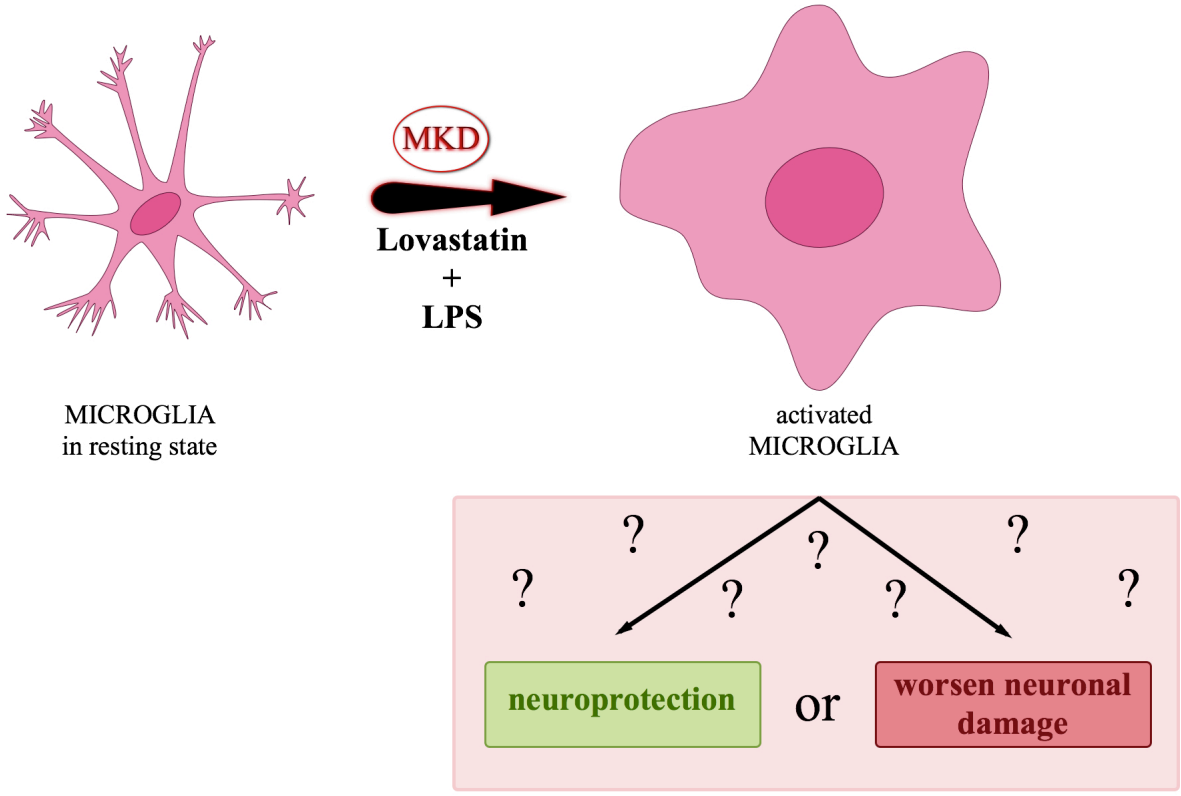

Figure 10: Microglia cells in resting state are morphologically characterized by many ramifications; instead, activated microglia cells are characterized by ameboid form. Block of mevalonate pathway and inflammatory stimulus, obtained by administration of Lovastatin and LPS, could cause microglia activation. Does microglia activation cause neuroprotection or worsen neuronal damage?

Background: microglial cells are considered the macrophages of central nervous system (CNS); microglial cell in CNS exist in resting state, also known ramified state. Under physiological condition, microglial cells perform several functions, including: monitoring brain, maintaining homeostasis, and they exerting neuro-protective and neurodegenerative actions [75].

Microglial cells are activated in many neurodegenerative disorders and during brain insult. Indeed, they begin a specific program that results in the gradual transformation of resting into an ameboid form, increasing the size of cell body and retracting their processes. This activation determines conformational change and also functional change: they begin to produce and secrete pro-inflammatory cytokines and chemokines [75,76].

Furthermore, activated microglial cells can cause heterogeneous responses according to the type of pathology, in fact, they can act on nearby neurons protecting them from injury, resulting in neuroprotection, or alternatively, they may induce neuronal damage or worsen the existing damage [77,78].

Mevalonate pathway block, in *in vitro* models obtained in monocyte cell line (RAW 264.7), induces an increase of cytokines release (in particular IL-1β that is an inflammatory MKD marker) and an increase of NALP3 expression, also in studies conducted in peripheral blood mononuclear cells from MKD patients [57,55].

Aim: understanding the consequences of microglial activation on neuronal cells is important to better comprehend MKD pathogenic mechanism and to propose novel therapeutic strategies based on the information disclosing the relationships between neuronal and microglial cells (Figure 10). Currently, there are little information about the CNS inflammation in MA patients, therefore, studies on this topic are fundamental to better understand the effects of the mevalonate pathway block in CNS.

Method: we evaluated microglial activation in *in vitro* model of MKD, and its interaction with neuronal cells. We used an *in vitro* co-culture model of microglial (BV-2) and neuronal (SH-SY5Y) cells (alona, transwell, direct contact) stimulated simultaneously with Lovastatin and LPS, mimicking the condition of MA patients exposed to pathogens. We evaluated programmed cell death, mitochondrial membrane potential, cytokines' release and cells' morphology modification.

Principal results: glial cells underwent an evident activation, confirmed by elevated pro-inflammatory cytokines release and modification in morphology. When glial cells were co-cultured with neurons, their activation caused an increase of programmed cell death also in neuron cells, only in direct contact co-culture.

Conclusions: contact co-culture between glial cells and neuronal cells seems to be a good model to study MA in *in vitro* model. Microglial activation is a direct consequence of mevalonate pathway block, which induces an additional increase of neuronal apoptosis. Therefore, we can hypothesize that the use of microglial activation blockers could prevent this additional neuronal death.

Ref: "Microglia activation and interaction with neuronal cells in a biochemical model of mevalonate kinase deficiency." Tricarico PM, Piscianz E, Monasta L, Kleiner G, Crovella S, Marcuzzi A. Apoptosis. 2015; 20(8):1048-55.
Pubmed link: http://www.ncbi.nlm.nih.gov/pubmed/26003816

CHAPTER 5

IN VIVO MODEL OF MKD

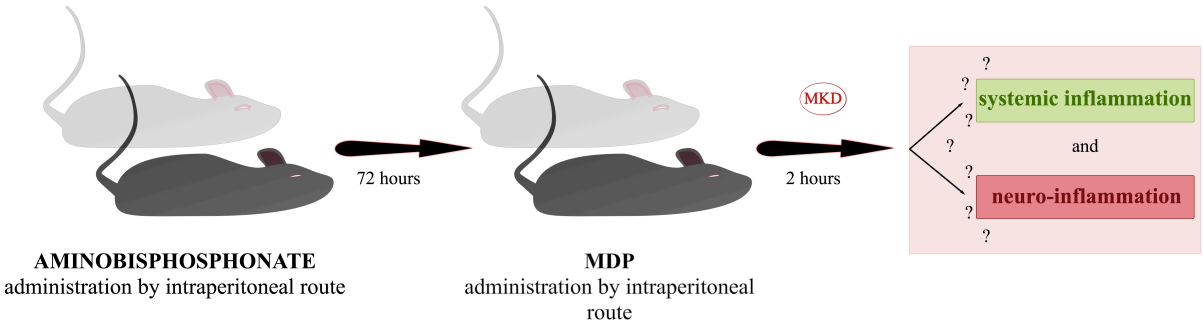

Figure 11: MKD *in vivo* mouse model is obtained by intraperitoneal administration of aminobisphosphonate for 72h and muramyldipeptide (MDP) for other 2h. Is biochemical *in vivo* model able to mimic MKD typical systemic inflammation and neuro-inflammation?

Background: at present, the only *in vivo* model that reproduces MKD inflammatory phenotypes is the one employing the biochemical model. This model is obtained by the administration of aminobisphosphonate, as alendronate, that inhibits farnesyl pyrophosphate synthase (FPPS), an enzyme of the mevalonate pathway downstream MK, and muramyldipeptide (MDP) to trigger strong inflammatory attack.

Previous experiments demonstrated that co-administration of alendronate plus MDP prompted an acute inflammation condition significantly greater than the alendronate alone; other *in vivo* studies did not use MDP but LPS, with similar results. Nevertheless, in our studies MDP was preferred because it was better tolerated than LPS by the animals, and because it better represents the mild stimulus capable to induce a MKD episode [69,79]. We preferred aminobisphosphonate administration in *in vivo* models, and not lovastatin as *in vitro* models, only for technical reasons: previous studies using aminobisphosphonate in *in vivo* models allowed us to simplify and consolidate an existing model [71-73]. Indeed, both compounds respect current pathogenic hypothesis, causing the decrease of isoprenoids and end products of the mevalonate pathway. Therefore, the use of statin or aminobisphosphonate for reproducing MKD phenotype is quite similar and the choice of one of them should not alter the results.

In many neurodegenerative diseases the role of neuro-inflammation is important, and manipulation of this inflammation may help to ameliorate clinical symptoms delaying disease's progression. Neuro-inflammation is connected to microglial activation [80,81]: as mentioned in chapter 4, we observed an important increase of NALP3 expression in activated microglial cells, which induces an increase of active IL-1β production.

Furthermore, there are no MKD *in vivo* models that exhibit the features of neurological dysfunction. Aminobisphosphonate is able to cross the blood-brain barrier.

Aim: assess the role-played in MKD by the inflammasome and the reliability of this *in vivo* model.

Method: we mimicked MKD using two different mice strains (BALB/c and C57BL/6), evaluating systemic inflammation and neuro-inflammation (Figure 11).

Principal results: biochemical *in vivo* model do not seem to vary in BALB/c and C57BL/6 mice strains, in fact both strains exhibited a general MKD-like systemic inflammation.

Conclusions: although with some limitation, this *in vivo* biochemical model appears robust and suitable for studying MKD. Additionally for the first time, we assessed inflammasome NALP3 activation in the central nervous system in *in vivo* model.

Ref: “Systemic and neuronal inflammatory markers in a mouse model of mevalonate kinase deficiency: a strain-comparative study.” Kleiner G, Celsi F, Tricarico PM, Zacchigna S, Crovella S, Marcuzzi A. In Vivo. 2013; 27(6):715-22.
Pubmed link: http://www.ncbi.nlm.nih.gov/pubmed/24292573

CHAPTER 6

DRUGS TREATMENT AND TEMPERATURE IN MKD

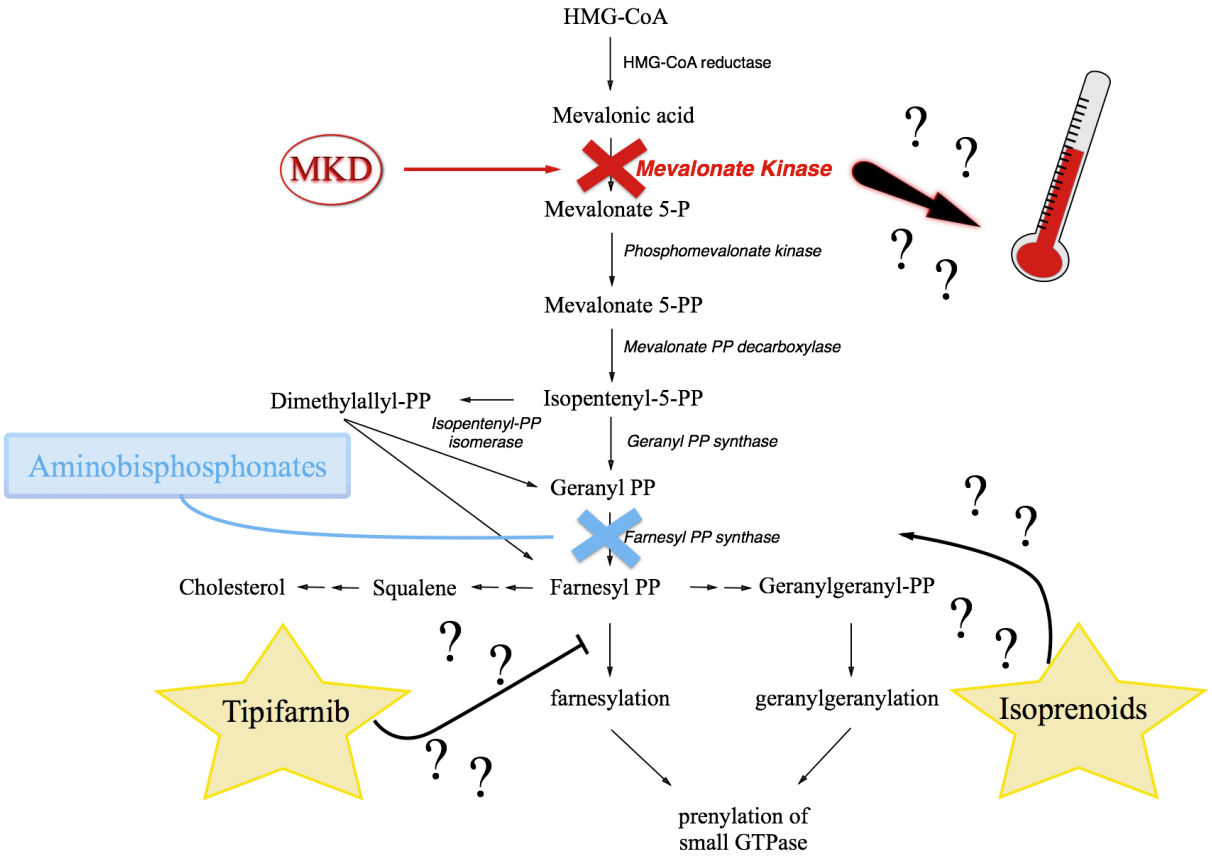

Figure 12: Is Mevalonate kinase activity appears influenced by temperature? Does temperature affect the ability of Tipifarnib (inhibitor of farnesylation) and isoprenoids to reduce the inflammatory phenotypes?

Background: MKD is a rare autoiflammatory disease and inflammatory phenotype seems to be caused by the activation of NALP3 inflammasome that triggers the release of IL-1β. This inflammatory mechanism is induced by the shortage of mevalonate- derived isoprenoid compounds.

In 2002 Houten SM and collaborators studied the effect of temperature on MK enzymatic activity. They realized that febrile temperatures produce a further decrease of MK activity and successive worsening of inflammatory phenotype. This decrease initiates a compensatory increase in the activity of HMG-CoA reductase, suggesting that MK becomes progressively rate-limiting itself [42].

The correlation between isoprenoids decrease and inflammatory phenotype is still not completely known. Our studies and studies conducted by van der Burgh R and collaborators highlight an important oxidized redox status caused by the mevalonate pathway block: this oxidized redox status subsequently involves the mitochondrial damage that subsequently activates NALP3 inflammasome [82].

Exogenous isoprenoids are able to act in the mevalonate pathway, bypassing the block and reducing the subsequent inflammation. Additionally, they have also an important anti-oxidative activity that could further improve the rescue of inflammation effects [83,84]. Another important molecule is Tipifarnib, an inhibitor of farnesylation, able to intervene in the mevalonate pathway reducing the farnesylation and consequently augmenting the geranylgeranylation pathway [85].

Aims: firstly, we evaluated the ability of Tipifarnib and geraniol, an exogenous isoprenoid, to reduce the inflammatory phenotypes and to elucidate how temperature can affect the success of these treatments; then, we evaluated the ability of three different exogenous isoprenoids to reduce the mitochondrial damage leading to oxidative stress and inflammatory phenotypes (Figure 12).

Method: we investigated the ability of geraniol and Tipifarnib to reduced the abnormal inflammatory response, in primary human monocytes obtained from MKD patients at different temperatures (37°C and 40°C).

The ability of isoprenoids compound to rescue MDK pathway and revert the inflammatory phenotype was assessed through the evaluation of cellular damage, mitochondrial dysfunction and inflammatory and oxidative markers, in murine monocyte/macrophage cell line).

Principal results: the febrile temperature in primary human monocytes from MKD patients induced an important increase of inflammatory markers. Fortunately, febrile temperature does not affect the ability of geraniol and Tipifarnib to decrease the inflammatory phenotypes.

Mevalonate pathway block induced mitochondrial damage, leading to oxidative stress, activation of NALP3 inflammasome and pro-inflammatory cytokines' release, driving cells to final apoptosis. Indeed, the administration of exogenous isoprenoids (such as lycopene, phytol and geranylgeraniol) induced an important rescue of mevalonate pathway and decrease inflammatory markers.

Conclusions: we evidenced the role of temperature in the modulation of the inflammatory events and suggested strongly considering this variable in future researches aimed at finding a treatment for MKD. Our findings confirm the hypothesis that inflammation is triggered by the shortage of isoprenoids.

Ref: "Temperature and drug treatments in mevalonate kinase deficiency: an ex vivo study." Tricarico PM, Kleiner G, Piscianz E, Zanin V, Monasta L, Crovella S, Marcuzzi A. Biomed Res Int. 2013; 2013:715465.
Pubmed link: http://www.ncbi.nlm.nih.gov/pubmed/24073415

"Block of the mevalonate pathway triggers oxidative and inflammatory molecular mechanisms modulated by exogenous isoprenoid compounds." Tricarico PM, Kleiner G, Valencic E, Campisciano G, Girardelli M, Crovella S, Knowles A, Marcuzzi A. Int J Mol Sci. 2014; 15(4):6843-56.
Pubmed link: http://www.ncbi.nlm.nih.gov/pubmed/24758928

CHAPTER 7

IN VITRO GENETIC MODEL OF MKD

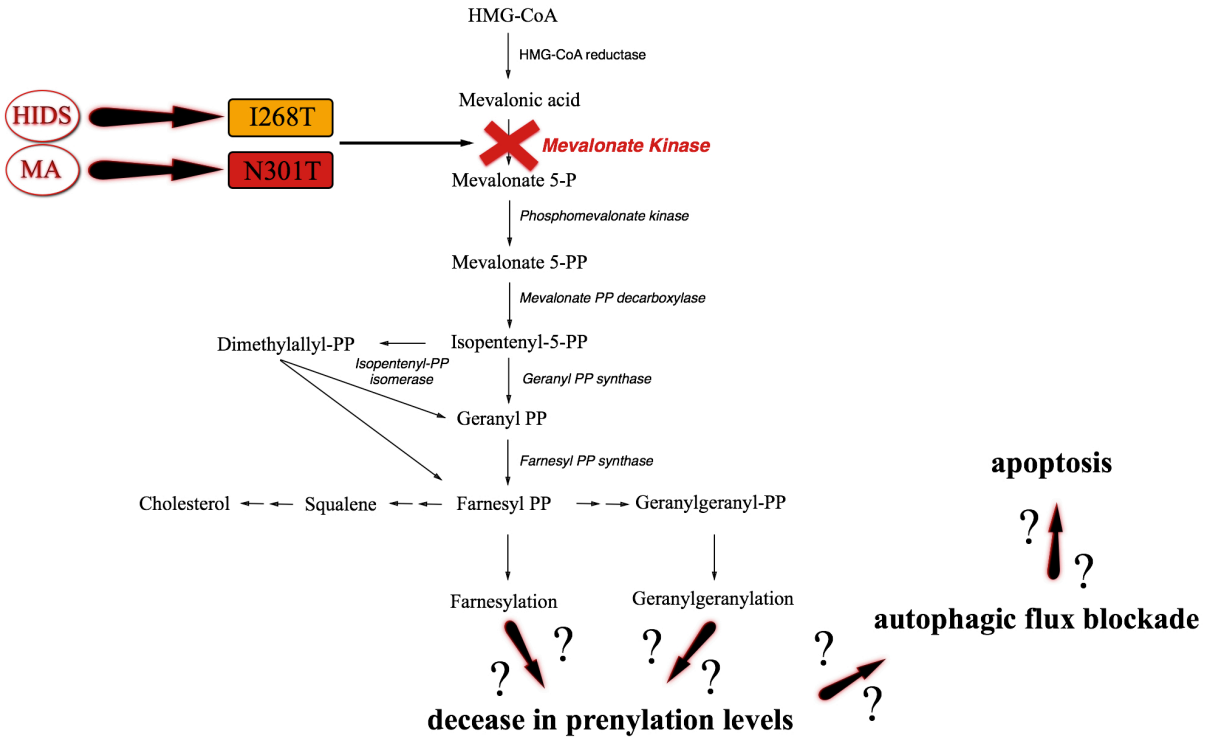

Figure 13: MKD *in vitro* genetic model is obtained by transient transfection with two different *MVK* mutations: I268T associated with Hyper-IgD syndrome (HIDS), and N301T typical of Mevalonic Aciduria (MA). Are Mevalonate Kinase mutations able to induce decrease in prenylation levels? Are the decreases in prenylation levels able to induce autophagic flux blockade that consequently causes apoptosis?

Background: currently, the only in vitro model of MKD is a biochemical one, obtained by administration of mevalonate pathway inhibitors. This model is obviously characterized by some limitations; indeed, it is not suitable to completely understand the differences between the mild form HIDS, and that more severe MA, and to clarify pathogenic mechanism related to the presence of different MVK genetic variations. All this considered, establishing a MKD genetic model is fundamental in order to clarify pathogenic mechanism related to the presence of specific MVK mutations (Figure 13).

van der Burgh R and co-authors in 2014, demonstrated that the block of HMG-CoA reductase (i.e. statin treatment) induces an increase of mitochondrial ROS in damaged mitochondria, and attenuates autophagosomal degradation. Therefore these mechanisms cause MKD typical hyper-secrection of IL-1β [82].

All this appears as a direct consequence of the mevalonate pathway block, which results in a decrease of geranylgeranyl pyrophosphate and farnesyl phyrophosphate, important for proteins prenylation.

Aim: shortage of prenylated proteins has been hypothesized as possible MKD pathogenic model. So, we studied the effects of I268T and N301T MVK mutations on proteins prenylation, autophagy and programmed cell death in SH-SY5Y neuronal cells.

Method: we obtained the *in vitro* genetic model of MKD using transient transfection of two different MKD mutations, namely I268T associated with HIDS, and N301T typical of MA: we evaluated prenylation levels, autophagic flux and apoptosis in neuroblastoma cell lines.

Principal results: we observed that *MVK* mutants' over-expression increased LC3 lipidation in SH-SY5Y, with concomitant p62 accumulation. An increased number of autophagosomes/cell (LC3 per cell) was detected in cells carrying *MVK* mutants with respect to those carrying wild type. SH-SY5Y with MVK mutants showed programmed cell death increase, being N301T the more relevant in terms of association with apoptosis.

Conclusions: we then hypothesize that mevalonate pathway impairment, due to MVK mutations, causes alteration of proteins prenylation and block of the autophagic flux; these alterations induce apoptosis. The presence of MA mutation N301T induces stronger apoptosis in neuronal cell line.

7.1 Introduction

Mevalonate kinase deficiency (MKD) is a monogenic disease showing a continuous phenotypic spectrum resulting into two distinct diseases: a mild one, Hyper-IgD Syndrome (HIDS, OMIM#260920), and a severe one, Mevalonic Aciduria (MA, OMIM #610377). HIDS is characterized by heterogeneous symptoms including recurrent fevers, cutaneous rash, aphthae, arthralgia, abdominal pain with diarrhoea and vomiting, while MA shows a more critical neurologic phenotype with psychomotor retardation and cerebellar ataxia [8].

Genetic variants in coding and non-coding mevalonate kinase gene (MVK, 12q24.11, NM_000431) are known to be associated with the disease (at present 203 variations have been reported, see INFEVERS http://fmf.igh.cnrs.fr/ISSAID/infevers/). However, the link between the genetic defects and MKD phenotypic spectrum remains to be elucidated, especially considering that the pathogenic mechanism at the basis of the disease is still elusive [86].

MVK encodes for mevalonate kinase (MK), a key enzyme of cholesterol pathway. This pathway is important for the production of cholesterol and other molecules, such as farnesyl-pyrophosphate and geranylgeranyl-pyrophosphate, crucially involved in proteins' prenylation. Proteins' prenylation is a key biochemical modification responsible for targeting and activating proteins to cell membranes [87] (see Figure 14a for complete description of the pathway).

One of the most accredited MKD pathogenic hypothesis relies on the lack of prenylated proteins: in patients' peripheral blood monocytes (PBMCs) the treatment with farnesol (FOH) or geranylgeraniol (GGOH), substrates for prenyl transferase, has been reported as being able to restore the normal response to inflammatory challenges [50]. This hypothesis was further confirmed in 2010, by demonstrating that activation of two small GTPase (RhoA and Rac1), which requires geranygeranyl pyrophosphate, is more disrupted in fibroblasts from MKD patients, compared to cells from healthy donor [88]. Van der Burgh and co-authors subsequently confirmed this pathogenic hypothesis and expanded it: they showed that treatment with simvastatin, a drug inducing mevalonat pathway blockade, (for a complete review, see [49]); GGPP is the analogue of cell-permeable GGOH and previous works have shown that GGOH reduces IL-1β secretion in PBMCs challenged with stat and it prevents statin cytotoxic effects in THP-1 cell line [89,90]. GGOH probably acts as a substrate for geranylgeranyl transferase, balancing isoprenoid shortage induced by statins treatment. So, considering that at present no model mimicking the effects of MVK mutations in vitro has been proposed, and only data derived from a biochemical model of MKD are available, we

established a transient expression model in neuroblastoma cell line (SH-SY5Y) using MVK coding plasmid carrying two different mutations: I268T (HGMD CM990888) associated to HIDS, and N301T (HGMD CM920489) typical of MA. Then we evaluated prenylation levels, autophagic flux and cell death in SH-SY5Y transfected model, with the aim of better disclosing the role of autophagy in MKD.

7.2 Methods

Cell Culture

SH-SY5Y neuroblastoma cells were chosen based on the clinical signs characterizing MA, the most severe phenotype of MKD. SH-SY5Y cells are a widely used model for neurodegenerative disease, possess neuronal features and are easy to be transfected. Cells, kindly provided by Prof. S. Gustincich (S.I.S.S.A.-I.S.A.S. Trieste, Italy) were cultured in MEM:F12 (Euroclone, Italy), supplemented with 10% fetal bovine serum (FBS, Euroclone, Italy), non-essential amino acid solution 1× (NEAA, Euroclone, Italy), 2 mM glutamine and penicillin streptomycin 1× solution (Sigma Chemical Co. Aldrich St. Louis, MO) and used between passages 4 to 10. Transfection was performed using Lipofectamine 2000 (Life Technologies, Carlsbad, USA) following manufactures' protocols.

Plasmids and site directed mutagenesis

MVK-expressing plasmid (Wild Type; RefSeq: NM_000431.1) (WT) was obtained from Origene (Rockville, USA) (RC201971). Two mutations were obtained: I268T and N301T, using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, California). Primers were designed using "PrimerX" (http://www.bioinformatics.org/primerx/index.htm). Mutagenesis was confirmed by direct sequencing. Plasmids GFP-RhoA and GFP-RhoAF were generously provided by G. Del Sal (LNCIB, Trieste, Italy).

GFP intracellular distribution

Intracellular distribution of GFP-RhoA, GFP-RhoAF and GFP-LC3 was evaluated using Zeiss Axioskop fluorescent microscope. Briefly, cells grown on glass coverslip and fixed in 4% paraformaldehyde were imaged using 40X objective and among 10-15 cell images for each experiments were taken. For GFP-RhoA and GFP-RhoAF parallel images for nuclear staining (DAPI; Life Technologies) were obtained. Composite images formed by GFP and DAPI signal were made using Fiji software (Schindelin et al., 2012) and overlap between the two signal was evaluated using JACoP plugin (Bolte and Cordeliers, 2006); if overlap coefficient was >0.60, intracellular distribution was considered "cytosolic". The reference

value for overlap coefficient was obtained using cells transfected only with reporter vectors and not treated (NT). For LC3-GFP distribution, the number of high intensity points ("LC3+ puncta") was counted for each cells9.

Western Blot

SH-SY5Y cells overexpressing MVK were lysed in RIPA buffer (10mM Tris-HCl pH 8.0, 1 mM EDTA, 1% Triton X-100, 140 mM NaCl). Cellular lysates were quantified using Bradford assay (Biorad, Hercules, USA); equal amounts of protein were loaded on 4-20% Tris-Tricine gels (Biorad) and transferred on nitrocellulose membrane (Biorad, Hercules, USA). Membranes were then incubated with primary antibodies and then developed with HRP-conjugated secondary antibodies and Clarity™ substrate (Biorad Hercules, USA).

Primary antibodies used in this study were: anti-MVK (Origene, Rockville, USA; TA313341); anti-hsp90 (SantaCruz, Dallas, USA); anti-p62 (SantaCruz, Dallas, USA); anti-GAPDH (SantaCruz, Dallas, USA).

Apoptosis Analysis

Apoptosis has been evaluated in SH-SY5Y cells expressing MVK with flow cytometry (Annexin V-FITC Apoptosis Detection Kit, Immunostep, Spain). Fluorescence was acquired with CyAn ADP analyzer and Summit software (Beckman Coulter, Brea, CA, USA), then analysed with FlowJo software (version 7.6, Treestar, Inc., St Ashland, OR, USA).

Statistical Analysis

Statistical significance was calculated using one-way ANOVA and Bonferroni post hoc test Correction. Analysis was performed using GraphPad Prism software (version 5.0, GraphPad Software, Inc., La Jolla, CA, USA).

7.3 Results

Firstly, we assessed MK protein levels in SH-SY5Y cells carrying WT MVK or mutant alleles (I268T and N301T). As shown in Figure 1b, SH-SY5Y cells showed similar exogenous MK protein levels.

We then determined the effect of MVK I268T and N301T mutations on proteins prenylation in SH-SY5Y, using GFP-RhoA-F to assess farnesylation, and GFP-RhoA to evaluate geranylgeranylation.

As shown in Figure 14c, GFP-RhoA-F or GFP-RhoA alone, in not transfected (NT) condition, localized in discrete compartments and plasma membranes; instead, in transfected cells with MVK, GFP-RhoA-F or GFP-RhoA localization becomes cytoplasmic.

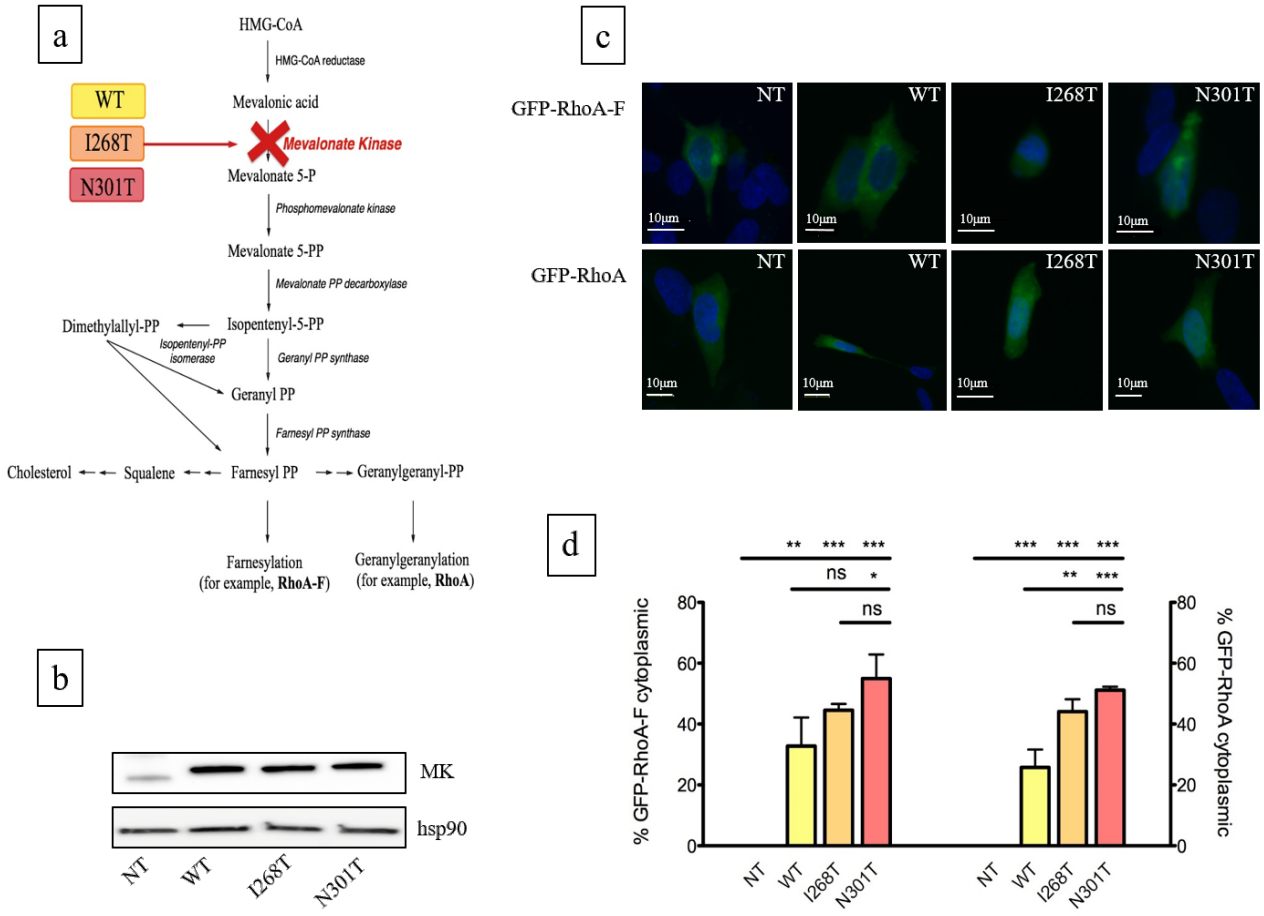

Figure 14 (a) Schematic representation of mevalonate pathway. Mevalonate Kinase enzyme is shown in red. In vitro MKD transient expression model, was established using MVK coding plasmid carrying two different mutations: I268T (orange), and N301T (red) and WT (Wild-Type of MVK, yellow). The principal product of mevalonate pathway is cholesterol, but this metabolic pathway is also important for the production of farnesyl-PP and geranylgeranyl-PP that generates respectively farnesylation and geranylgeranylation of small GTPase, among other proteins. (b) Evaluation of MK protein levels with western blot in SH-SY5Y cells carrying WT MVK or mutant alleles (I268T and N301T). Hsp90 is reference protein. 24h after seeding, cells were transfected with Lipofectamine 2000 for 48h with pCMV-6 plasmid, containing MVK wild type and mutated sequences. Transfected MVK presents a slightly higher molecular weight, due to the presence of myc-tag in pCMV-6 plasmid; endogenous protein is reduced due to unknown feedback mechanism: preliminary experiments shows an indirect relationship between levels of overexpressed MVK and endogenous protein (not shown). (c) SH-SY5Y transiently transfected with GFP-RhoA-F or GFP-RhoA. GFP–RhoA-F with a farnesylation consensus sequence is sensible to farnesylation; indeed, GFP–RhoA with a geranylgeranylation consensus sequence is sensible to geranylgeranylation. Images were acquired by Zeiss Axioskop fluorescent microscope and the composite imagines formed by GFP and DAPI signal were made using Fiji software. Representative images are shown. I268T and N301T demonstrate the typical cytosolic distribution. Scale bars, 10µm. (d) Percentages of cells with cytoplasmic distribution of GFP-RhoA-F and with cytoplasmic distribution of GFP-RhoA according to MVK sequences carried are reported. Data are derived from n = 3 independent experiments and 15 cell images for each experiment were taken. 24h after seeding, cells were transfected with Lipofectamine 2000 for 48h with pCMV-6 plasmid, containing MVK wild type and mutated sequences. SH-SY5Y cells transiently expressing different MVK mutations were co-transfected with GFP-RhoA-F or GFP-RhoA. NT= not transfected cells, WT=Wild-Type MVK, I268T=MVK mutated I268T, N301T=MVK mutated N301T. Statistical significance was calculated using one-way ANOVA and Bonferroni post-test correction. *P<0.05; **P<0.01, ***P<0.001, ns: not significant.

In transfected cells with MVK mutants, we observed a significant increase of the number of cells with GFP-RhoA-F and GFP-RhoA cytoplasmic localization when compared with NT condition (Figure 14d): in N301T mutation, we observed a significantly increase of cells with

GFP-RhoA-F and GFP-RhoA cytoplasmic localization if compared with WT (% of GFP-RhoA-F cytoplasmic distribution: N301T 54.94±7.92, $p<0.05$; WT 32.79±9.39) (% of GFP-RhoA cytosolic distribution: N301T 51.19±1.08, $p<0.001$; WT 25.75±5.87); instead in I268T mutation the increase becomes significant only for the GFP-RhoA localization when compared to WT (% of GFP-RhoA cytosolic distribution: I268T 44.10±4.07, $p<0.01$; WT 25.75±5.87) (Figure 14b).

We then investigated if I268T and N301T mutants could alter autophagy in SH-SY5Y. During autophagy, autophagosomes engulf cytosolic cargos and fuse with lysosomes to complete the degradation process [91]. Thus, we firstly investigated if MVK mutants could modify the number of autophagosomes.

In NT conditions, cells displayed diffuse cytoplasmic GFP-LC3; instead, the transfection with MVK mutants triggered the formation of numerous autophagosomes/cell (LC3+ "puncta" per cell) in SH-SY5Y cells (Figure 15a) (Number of autophagosomes/cell (LC3+ "puncta" per cell); WT 5.43 puncta/cell ±0.59, not significant when compared to NT; I268T 9.88 puncta/cell±1.23, $p<0.05$ when compared to WT; N301T 9.93 puncta/cell ±1.17, $p<0.05$ when compared to WT; NT 6.82 puncta/cell ±0.95). To investigate whether the elevated levels of LC3 lipidation induced by MVK mutants are due to increased formation of autophagosome or to decreased degradation, we measured the levels of the autophagic marker p62/SQSTM1.

p62 levels significantly increased in SH-SY5Y cells with expression of MVK I268T and N301T mutants if compared with NT (p62 relative density: I268T 1.46±0.008, $p<0.05$; N301T 1.46±0.14, $p<0.05$; NT 1) (Figure 15c/d).

SH-SY5Y transfected with MVK WT or I268T or N301T mutations, showed augmented PCD; these results are also graphically evident in flow cytometric plots (Figure 15e). Although just the overproduction of MVK protein in WT causes statistically significant increase of PCD with respect to NT conditions, in the presence of N301T this PCD increase was more significant (PCD: WT 29.06±2.05, $p<0.05$; I268T 31.17±1.70, $p<0.05$; N301T 33.57±0.81, $p<0.01$; NT 17.76±3.46) (Figure 15f).

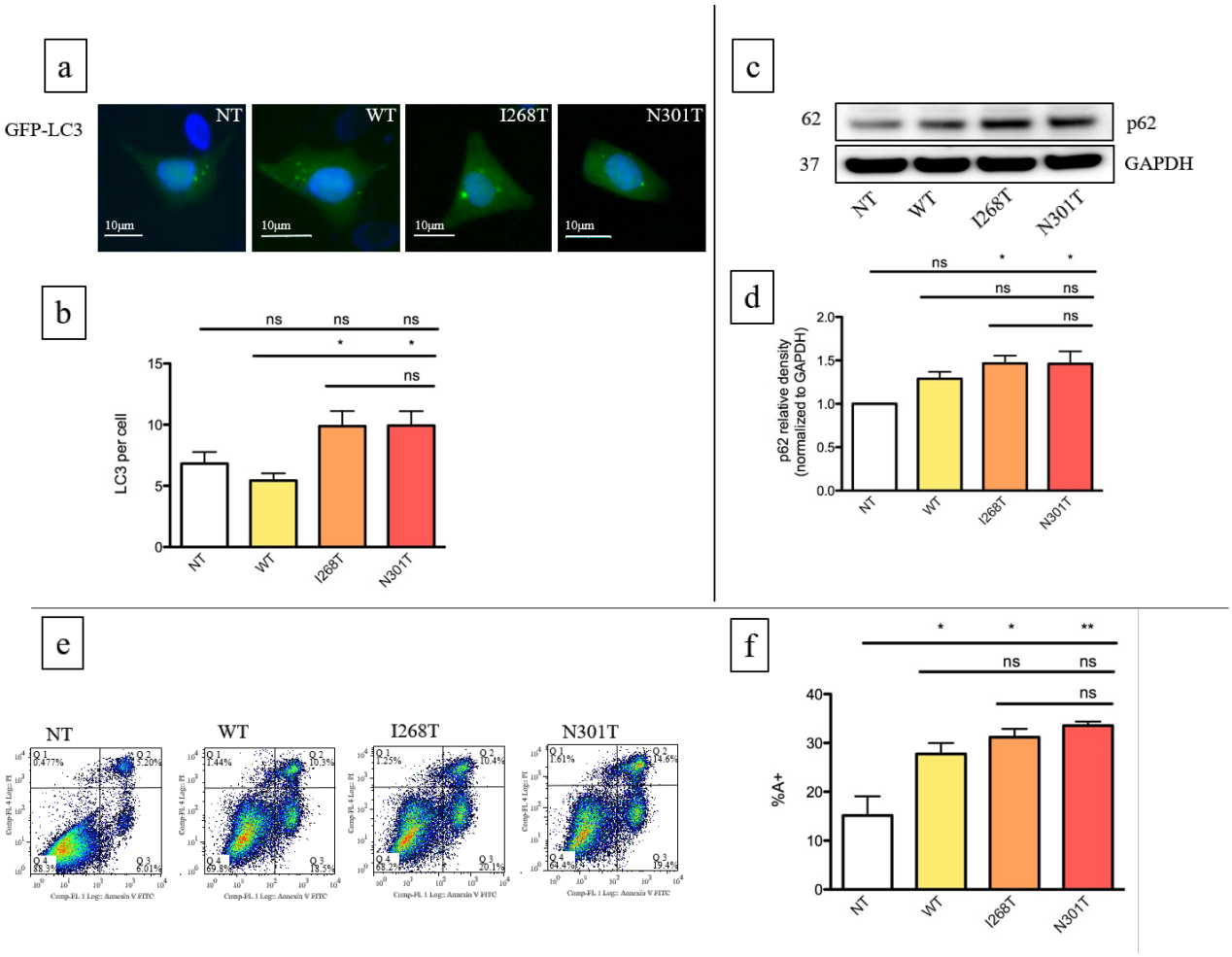

Figure 15 (a) Representative images showing activation of autophagy in SH-SY5Y transiently transfected with GFP-LC3 and WT MVK and different MVK mutations (I268T and N301T). Images were acquired by Zeiss Axioskop fluorescent microscope and the composite imagines formed by GFP and DAPI signal were made using Fiji software. Scale bars, 10µm. (b) Number of autophagosomes/cell (LC3+ "puncta" per cell) in SH-SY5Y cells. Data are derived from n = 3 independent experiments and 15 cell images for each experiment were taken. SH-SY5Y cells transiently expressing pCMV-6 plamid with MVK mutations were transfected with GFP-LC3. (c) Representative western blot of p62; GADPH is the reference protein. (d) Relative levels of p62 protein quantified with western blot in SH-SY5Y cells carrying WT MVK or mutant alleles (I268T and N301T). p62 is significantly increased in cells overexpressing mutant alleles (I268T and N301T) in comparison with cells overexpressing WT MVK. GADPH is the reference protein. (e) Representative flow cytometry plots of SH-SY5Y cells carrying WT MVK or mutant alleles (I268T and N301T) stained with Annexin V and Propidium Idide. Numbers in the corners of quadrants represent the percentage of the total number of cells. (f) Apoptosis has been evaluated in SH-SY5Y cells expressing pCMV-6 plasmid with MVK mutations, with flow cytometry. Bars represent the average of % Annexin positive cells (%A+) ± standard deviation (S.D.) of three independent experiments. Fluorescence was acquired with CyAn ADP analyzer and Summit software, then analysed with FlowJo software. 24h after seeding, cells were transfected with Lipofectamine 2000 for 48h with pCMV-6 plasmid, containing MVK wild type and mutated sequences. NT= not transfected cells, WT=Wild-Type MVK, I268T=MVK mutated I268T, N301T=MVK mutated N301T. Statistical significance was calculated using one-way ANOVA and Bonferroni post-test correction. *P<0.05; **P<0.01, ns: not significant.

7.4 Discussion

Our findings suggest that mevalonate pathway impairment, due to MVK mutations, (I268T and N301T) causes alteration of prenylation mechanism, followed by induction of autophagy that never reach successful completion; these changes in cellular metabolism induce

apoptosis, significantly increased in the presence of N301T mutation associated with MA leading us to hypothesize a functional correlation with the most severe phenotype of the disease.

Our results are in accordance with the assumption that defects in prenylation may trigger impairment of autophagy [20]. Indeed, our work confirms and expands results obtained in monocytes from HIDS patients [92]. In neuronal cells, MA mutation could cause a statistically significant further decrease in prenylated protein level, compared to HIDS related mutation. This could lead to an additional impaired autophagy and consequently a greater apoptosis, thus explaining the more severe phenotype of MA. The crosstalk between autophagy and apoptosis pathways has been demonstrated in different models, for a review see [93].

Autophagy is a complex process, multiple pathways are known to control this process also mediated by numerous small GTPases, necessitating prenylation to correctly work. Although preliminary, the present work suggests a tight link between alteration in autophagic flux and mevalonate pathway dysfunction, followed by cell death [49,94]. A recent study in rheumatoid arthritis demonstrated that increasing autophagic flux could be beneficial toward lowering markers of inflammation and a previous study also demonstrated that inhibition of mTor pathway could ameliorate symptoms of patients suffering of rheumatoid arthritis [95,96]. Indeed our findings suggest that mevalonate pathway disturbances could be responsible for autophagic flux blockade. Van der Burgh and co-authors have previously shown that treatment of HIDS patients' monocytes with inhibitor of mTor pathway, after LPS challenge, slightly decrease IL-1β secretion, although not with levels similar to control [20]. These data showed how autophagy is a complex process and different aspects still need to be evaluated.

MKD is an "orphan" disease, treatment is only supportive and there is no evidence-based therapeutic protocol. It will then be crucial to identify the small GTPases responsible of autophagy impairment aimed at uncovering novel possible treatment strategies for both HIDS and MA. A recent work identified the axis RhoA-Rac1 as important for IL-1β hypersecretion, with inactivation of RhoA being a signal for IL-1β "priming" [82]. Delving deeper into the complex interplay of these, among others, small GTPase could represent a step forward toward a better understanding of the complex molecular mechanism underlying MKD.

Autophagy flux blockade appears to be crucial in different pathologies, and a recent work in TRAPS (TNFR-associated periodic syndrome) patients demonstrated how mutation in TNF-α receptor causes autophagy blockade and ROS increase followed by IL-1β augmented

secretion [97]. A more recent review from the same authors hypothesizes a common mechanism between pathologies that have increased inflammation (TRAPS, Cystic Fibrosis, Alzheimer's Disease, Parkinson's Disease); this common mechanism could be the autophagy's blockade leading to increased ROS and release of IL-1β. We have previously demonstrated that a novel mutation in MVK (R277G) distorts the active site, rendering it less sensible to inhibition; recently other authors have systematically analysed MVK mutations, finding that changes in aminoacidic sequence modify its stability and propensity to aggregate [98-99]. From these works is possible to hypothesize a mechanism similar to the one envisaged for TRAPS: mutant MVK is more unstable and prone to aggregate, blocking autophagic flux and setting off inflammation. Indeed more studies are needed to further expands our knowledge of these mechanisms and provide novel target for a therapeutic intervention. So our study represents a starting point towards a reconsideration of MKD known disease mechanisms: further experiments are needed to determine which proteins, between the small GTPases family, are the primary actors of this dysfunction.

CHAPTER 8

NEW PATHOGENIC HYPOTHESIS OF MKD

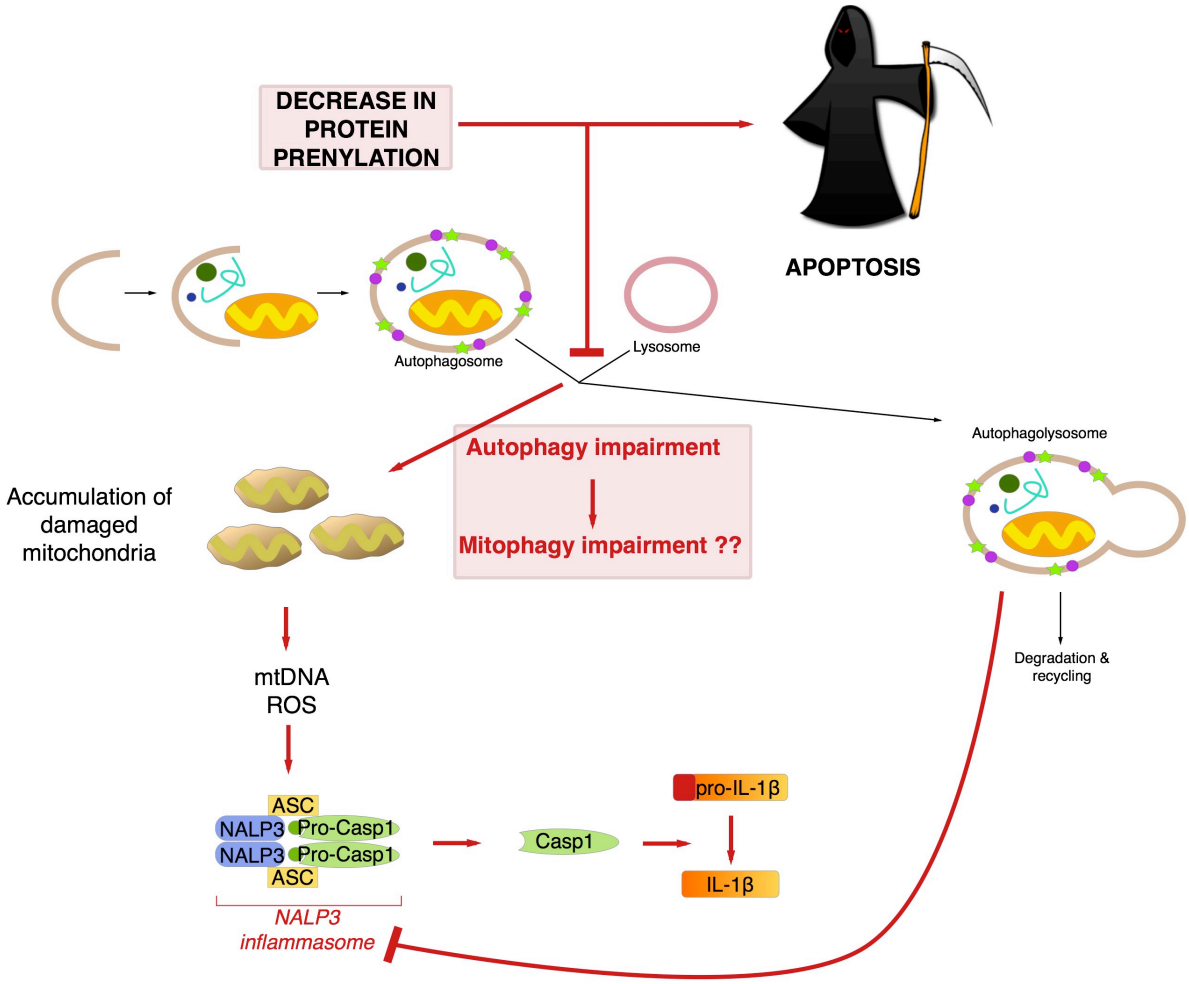

Figure 16: New MKD pathogenic hypothesis: decrease in protein prenylation induces apoptosis. Apoptosis is caused by autophagy impairment and in particular by mytophagy impairment. Indeed, mitophagy impairment induces accumulation of damaged mitochondria, which cannot be recycled, causing release of mitochondrial DNA (mtDNA) and ROS. All this events prompt NALP3 inflammasome activation with consequent cleavage and activation of IL-1β.

Background: the mevalonate pathway, crucial for cholesterol synthesis, plays a key role in multiple cellular processes. Deregulation of this pathway is also correlated with diminished protein prenylation, an important post-translational modification necessary to localize certain proteins, such as small GTPases, to membranes. Mevalonate pathway blockade has been linked to mitochondrial dysfunction: especially involving lower mitochondrial membrane potential and increased release of pro-apoptotic factors in cytosol. Furthermore a severe reduction of protein prenylation has also been associated with defective autophagy, possibly causing inflammasome activation and subsequent cell death.

Hypothesis: a mechanism in which defective autophagy fails to remove damaged mitochondria, resulting in increased cell death. This mechanism could play a significant role in Mevalonate Kinase Deficiency, an autoinflammatory disease characterized by a defect in Mevalonate Kinase, a key enzyme of the mevalonate pathway. Patients carrying mutations in the MVK gene, encoding this enzyme, show increased inflammation and lower protein prenylation levels.

Conclusions: there is a strong correlation between mevalonate pathway defects, mitochondrial dysfunction and defective autophagy, as well as inflammation. This correlation could represent a new pathogenetic hypothesis of Mevalonate Kinase Deficiency (Figure 16).

Ref: "Mevalonate Pathway Blockade, Mitochondrial Dysfunction and Autophagy: A Possible Link." Tricarico PM, Crovella S, Celsi F. Int J Mol Sci. 2015 Jul 15;16(7):16067-84.
Pubmed link: http://www.ncbi.nlm.nih.gov/pubmed/26184189

CONCLUSION AND PERSPECTIVES

The purpose of PhD was better understanding the pathogenic mechanism of Mevalonate Kinase Deficiency, evaluating the neuro-apoptotic and neuro-inflammatoy mechanisms occurring in the disease or in models mimicking MKD.

Overall, the results reported in this thesis (Figure 17) indicate that:

- *GRID2* has been identified using an exome chip in 22 MKD patients as a potential MKD modifier gene, having been already described as associated, when mutated, to cerebellar atrophy and ataxia; nevertheless, the involvement of *GRID2* variants in MA patients deserves further investigation. Indeed, it surely represents a first step towards clarifying the influence of host genetic background other than *MVK* in MKD, as well as to better understanding genotype-phenotype correlation. Next step will be to perform whole exome analysis of patients with severe phenotype MA, not investigated in this thesis due to the rarity of this disease and the unavailability of samples (Chapter 2).

- Biochemical block of mevalonate pathway induced apoptosis in neuronal cells following the mitochondrial pathway. We observed a caspase-9 and caspase-3 dependent apoptosis; however, we also demonstrated that caspase-1 play a role in this mechanism. So in neuro-infammation caused by block of mevalonate pathway, we hypothesize a balance between apoptosis and pyroptosis, a caspase-1 dependent programmed cell death (Chapter 3).

- Microglial activation is a direct consequence of mevalonate pathway block, which induces an additional increase of neuronal cell death. Therefore, we hypothesise that the use of microglial activation blocker could prevent this additional neuronal death. Further studies will be carried out to confirm this hypothesis (Chapter 4).

- Systemic and neuronal inflammations are observed in biochemical *in vivo* model obtained in two different mice strains. For the first time, we assessed inflammasome NALP3 activation in the central nervous system in *in vivo* model: we did not observe significant differences between the two strains of mice. In this way, we have consolidated our *in vivo* model, which can be used to test new drugs for MKD treatment, also evaluating the impact on the central nervous system (Chapter 5).

- Mevalonate pathway block induced mitochondrial damage, leading to oxidative stress and pro-inflammatory cytokines' release, driving cells to final apoptosis. This seems to be direct

consequence of isoprenoid compounds shortage. Indeed, the administration of exogenous isoprenoids induced an important improvement of all these inflammatory features.

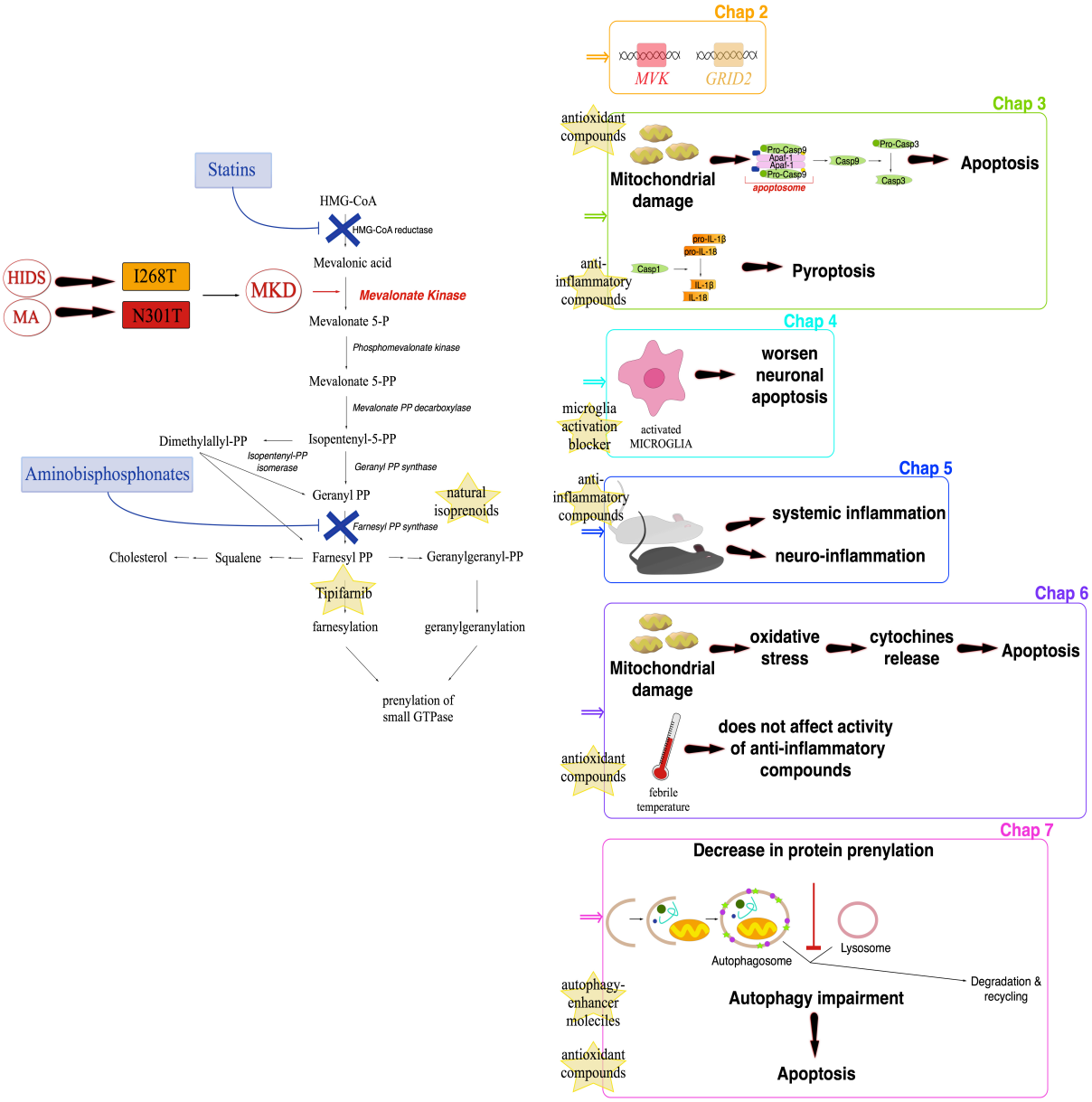

Figure 17: On the left: schematic representation of mevalonate pathway. Compounds used in the experiments performed during the PhD are indicated alongside the pathway: statins and aminobisphosphonates were used to obtain MKD biochemical model. MKD genetic model is obtained by transient transfection with two different *MVK* mutations: I268T associated with Hyper-IgD syndrome (HIDS), and N301T typical of Mevalonic Aciduria (MA). On the right: schematic representation of results reported in all chapters thesis. Chapter 2: GRID2 has been identified a potential MKD modifier gene. Chapter 3: block of mevalonate pathway induced mitochondrial damage that causes caspase-9 and caspase-3 dependent apoptosis. Caspase-1 plays a role in this mechanism, probably promoting pyroptosis. Chapter 4: mevalonate pathway block induced microglial activation that prompt an additional increase of neuronal apoptosis. Chapter 5: biochemical *in vivo* model obtained in BALB/c and C57BL/6 mice strains are characterized by systemic and neurological inflammations. Chapter 6: mevalonate pathway block induced mitochondrial damage, leading to oxidative stress and cytokines' release, driving cells to apoptosis. Febrile temperature does not affect the success of Tipifarnib and natural isoprenoid (represented as a stars alongside the pathway), compounds able to decrease the inflammatory phenotypes. Chapter 7: alteration of mevalonate pathway causes decrease in protein prenylation levels that induce autophagy impairment, leading cells to final apoptosis. In balloon stars are shown the possible compounds able to modify the phenotype observed, caused by mevalonate pathway alteration.

Temperature has a crucial role in the modulation of inflammatory events correlated to MKD: a febrile temperature in primary human monocytes from MKD patients induced an important increase of inflammatory markers. Fortunately, febrile temperature does not affect the success of compounds (geraniol and Tipifarnib) able to decrease the inflammatory phenotypes (Chapter 6).

- *MVK* mutations cause an alteration in autophagic flux leading cells to final apoptosis, in *in vitro* (neuronal cells) genetic model of MKD. Additionally, N301T mutation associated with severe form of MKD, induces a higher percentage of cells in apoptosis, compared to I268T, indicating a more severe impact on neuronal surviving. Autophagic flux blockade could be caused by mevalonate pathway disturbance: indeed, we measured a decrease of farnesyl pyrophosphate and geranylgeranyl pyrophosphate, crucially involved in prenylation of proteins in this genetic model. The link between autophagic flux blockade and decrease in prenylated protein levels are the small GTPases; these small GTPases cannot perform their functions in completing the autophagic process if not prenylated. Further studies are necessary to identify the small GTPases responsible of this autophagy impairment (Chapter 7).

- All results obtained enable us to formulate a new MKD pathogenetic hypothesis, based on mitophagy, which links mitochondrial damage and autophagy flux blockade. Therefore, decrease in protein prenylation induces autophagy/mitophagy blocking that causes accumulation of damaged mitochondria, which cannot be recycled. Accumulation of damage mitochondria induces oxidative stress, inflammation and subsequent cell death.

Future studies are needed to further confirm these findings. Nevertheless, this new pathogenic mechanism suggests that the use of antioxidant or autophagy-enhancer molecules could possibly recover MKD phenotypes (Chapter 8).

In the last years, autophagy defect was identified in many autoinflammatory diseases, characterized by an accumulation of IL-1β. Therefore, this strong correlation between autophagic flux blockade and inflammation, with increased production of IL-1β, may be a common mechanism for several auto-inflammatory diseases.

For all these reasons, autophagy should be considered when trying to design novel therapeutic strategies to fight cell death in patients with several autoinflammatory disease, in particular MKD disease.

Perspectives:

- **perform whole exome analysis of patients with severe phenotype MA for clarifying the influence of host genetic background other than MVK in MKD;**
- **consolidate MKD *in vivo* model to recreate MA model that resembles the**

neurological features of the human disease as closely as possible;

- **establish stable MKD clones in neuronal and microglial cells overexpressing different MVK mutations for better understand the phenotypes/genotypes correlation. We will use these clones to further confirm the autophagy impairment and to evaluating mitophagy impairment. Furthermore, we will evaluate the ability of different autophagy-enhancer molecules (such as Rapamycin and Everolimus) to revert this impairment, and of different antioxidant compound (such as MitoQ, MitoTempo and MitVitE) to block mitochondrial damage.**

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