Intracellular cholesterol accumulation and coenzyme Q\textsubscript{10} deficiency in cellular models of familial hypercholesterolemia

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Abstract

Familial hypercholesterolemia (FH) is an autosomal co-dominant genetic disorder characterized by elevated low-density lipoprotein (LDL) cholesterol levels and increased risk for premature cardiovascular disease. Here, we examined HF pathophysiology in skin fibroblasts derived from FH patients harbouring heterozygous mutations in the LDL-receptor. Fibroblasts from FH patients showed a reduced LDL-uptake associated with increased intracellular cholesterol levels and coenzyme Q\textsubscript{10} (CoQ\textsubscript{10}) deficiency suggesting dysregulation of the mevalonate pathway. Secondary CoQ\textsubscript{10} deficiency is associated with increased ROS production, mitochondrial depolarization and mitophagy activation in FH fibroblasts. Persistent mitophagy alters autophagy flux and induced inflammasome activation and increased production of cytokines by mutant cells. All the pathological alterations in FH fibroblasts were also reproduced in a human endothelial cell line by silencing the LDL-receptor. Both increased intracellular cholesterol and mitochondrial dysfunction in FH fibroblasts were partially restored by CoQ\textsubscript{10} supplementation. Dysregulated mevalonate pathway in FH including increased expression of cholesterogenic enzymes and decreased expression of CoQ\textsubscript{10} biosynthetic enzymes were also corrected by CoQ\textsubscript{10} treatment. Reduced CoQ\textsubscript{10} content and mitochondrial dysfunction may play an important role in the pathophysiology of early atherosclerosis in FH by increasing ROS production and inflammation in blood vessels. The diagnosis
of CoQ₁₀ deficiency and mitochondrial impairment in FH patients may also be important to establish early treatment with CoQ₁₀.

Key words: Familial Hypercholesterolemia, LDL-R, cholesterol, coenzyme Q₁₀ deficiency, mitochondria dysfunction, inflammasome, mitophagy.

Running Title: Mitochondrial dysfunction in Familial Hypercholesterolemia
INTRODUCTION

Familial Hypercholesterolemia (FH) is an autosomal dominant disorder caused by genetic mutations\(^1\). In most cases, these mutations are located in the coding region of the low density lipoprotein receptor (LDL-R)\(^2\) which produces defective LDL-R that does not localize to the plasma membrane and consequently does not properly bind the LDL cholesterol (LDL-C) particle or fails to internalize into the cell after binding\(^3\). Although this is by far the most common mutation, there are other genes involved such as Apolipoprotein B\(^4\), Pro-protein Convertase Subtilisin/Kexin 9 (PCSK9)\(^5\), LDL Receptor Adaptor Protein 1 (LDLRAP1)\(^6\) or Apolipoprotein E\(^7\). Its prevalence varies from 1 in 500 in heterozygous FH to 1 in a million in homozygous FH\(^8\). It has been described that those mutations result in severely elevated serum cholesterol concentrations, premature atherosclerosis, cardiovascular morbidity and mortality\(^9\).

The mevalonate pathway converts acetyl-CoA to farnesyl diphosphate, and produces precursors for several metabolically important molecules as well as physiologically important end-products, including: 1) isopentenyl adenosine (important for t-RNA modification); 2) coenzyme \(Q_{10}\) (CoQ\(_{10}\)) (an antioxidant also important in the electron transport chain in mitochondria); 3) farnesyl diphosphate and geranylgeranyl diphosphate (lipid moieties that can be added to proteins to promote membrane association); 4) dolichol and dolichol-phosphate (important for protein glycosylation); and 5) cholesterol (precursor for bile acids and steroid hormones)\(^\text{10}\). This route is highly
regulated by endogenous metabolites such as oxysterols, squalene oxide, farnesol and its derivatives. As cholesterol levels are higher in FH patients, it has been reported that CoQ\textsubscript{10} levels can be also affected, as both compounds are synthetized in the same pathway. CoQ\textsubscript{10} is the predominant form of ubiquinone in humans and a component of the mitochondrial respiratory chain. It is widely known that CoQ\textsubscript{10} is an important antioxidant distributed among cellular membranes and that it plays a crucial role in cellular metabolism.

It has been suggested that CoQ\textsubscript{10} deficiency, caused either by a primary or secondary deficiency, is likely to be a useful mitochondrial dysfunction marker. In fact radical oxygen species (ROS) production, oxidative stress, reduction in ATP levels, diminished membrane potential and low activity of mitochondrial complexes have been demonstrated to be common consequences of dysfunctional mitochondria in human fibroblasts and muscles carrying CoQ\textsubscript{10} deficiency. In order to maintain cellular homeostasis, dysfunctional mitochondria need to be removed by a selective autophagy mechanism known as mitophagy. However, persistent and extensive mitophagy can impair cell bioenergetics and induce cell death.

Dysfunctional mitochondria have been pointed out as a risk factor to develop atherosclerosis due to its importance for cell survival and for its connection with inflammasome activation. Atherosclerosis is an inflammatory disease characterized by the accumulation of lipids and other components in arteries. FH patients are likely to suffer from premature
atherosclerosis not only due to lipid accumulation but also by mitochondria
damage\textsuperscript{22, 26}. On the other hand, CoQ\textsubscript{10} therapies have been reported to
have beneficial effects in both mitochondrial\textsuperscript{14, 27} and hyperlimidemic patients
\textsuperscript{28} and cardiovascular diseases in which atherosclerosis plays a major role\textsuperscript{14}.

The aim of this study was to examine how LDL-R mutations affect
mevalonate pathway and secondarily CoQ\textsubscript{10} content, mitochondrial function,
autophagy and inflammasome activation. Finally, we examined if the
treatment with CoQ\textsubscript{10} improved FH fibroblasts pathophysiology.
RESULTS

Cholesterol accumulation in fibroblasts derived from patients harbouring LDL-R mutations

In order to determine whether there was any evidence of defective LDL-R function in fibroblasts derived from FH patients, an *in vitro* LDL uptake assay was performed. Fluorescent labeled LDL complexes were used to evaluate the incorporation of cholesterol mediated by LDL-R in control and patient fibroblasts. All patient fibroblasts showed, as expected, a decrease of 60±10% of LDL uptake compared to control fibroblasts ([Fig. 1A and 1B](#)). Decreased LDL uptake was associated with reduced expression levels of LDL-R in FH fibroblasts assessed by Western blotting ([Fig. 1C and 1D](#)) and immunofluorescence microscopy ([Supplementary Fig. 1](#)).

Given that FH fibroblasts showed decrease LDL-cholesterol uptake, we next examined intracellular lipids in control and patient fibroblasts by red oil staining, a lysochrome commonly used to stain neutral lipids including esterified cholesterol. A significant increase in red oil staining was found in patient fibroblasts ([Fig. 1E and 1F](#)). Intracellular lipid accumulation was confirmed by electron microscopy examination that showed the presence of lipid droplets inside patient fibroblasts ([Fig. 1G and 1H](#)). To characterize lipid accumulation, cells were stained with Filipin, a highly fluorescent polyene macrolide antibiotic which binds specifically to unesterified cholesterol but not to esterified sterols. Filipin staining was notably increased in patient fibroblasts indicating increased cholesterol content in patient cells ([Fig. 1I](#)).
and 1J). Mevalonate pathway is an important metabolic pathway which plays a key role in multiple cellular processes by synthesizing sterol isoprenoids, such as cholesterol, and non-sterol isoprenoids, such as dolichol, heme-A, isopentenyl tRNA and CoQ_{10}. It has been suggested that there is a relation between cholesterol and CoQ_{10} levels due to the fact that both compounds are synthesized in the same pathway. In order to determine if mevalonate pathway was working properly in FH fibroblasts, we decided to examine de novo synthesis of two of the main final product of this pathway, cholesterol and CoQ_{10}. Radioactive labelled mevalonate was incorporated to cells and cholesterol and CoQ_{10} levels were examined by thin layer chromatography. The radioactivity incorporated in cholesterol was significantly increased (Fig. 2A) while the radioactivity incorporated in CoQ_{10} was significantly reduced in FH patients (Fig. 2B). These findings made us suspect that elevated cholesterol biosynthesis might have induced increased cholesterol levels and a secondary deficiency of CoQ_{10}. To assess this hypothesis, cholesterol and CoQ_{10} levels were measured in control and FH fibroblasts by HPLC. Cholesterol levels were marked increased (Fig. 2C) and CoQ_{10} levels were significantly reduced (Fig. 2D) in patient fibroblasts.

To further examine the mechanism of dysregulated mevalonate pathway in FH, the expression levels of proteins involved in cholesterol and CoQ_{10} biosynthesis regulation were analyzed by Western blotting. The mature form of transcription factor SREBP-2 and SREBP-1 which regulates the protein expression of key enzymes involved in cholesterol and lipid biosynthesis were increased in FH fibroblasts (Supplementary Fig. 2 and
Accordingly, the expression levels of 3-hidroxi-3-metil-glutaril-CoA reductasa (HMGCR), lanosterol syntase and squalene syntase were upregulated. Concomitantly, FH fibroblasts showed reduced expression of proteins involved in CoQ\textsubscript{10} biosynthesis (Coq1, Coq2 and Coq7) (Supplementary Fig. 2, 3A and 3B). Correspondingly, up-regulated expression of cholesterogenic enzymes and down-regulated expression of CoQ\textsubscript{10} biosynthetic enzymes were accompanied by increased expression of cholesterogenic transcripts and decreased expression of COQ genes (Supplementary Fig. 3C).

**Mitochondrial dysfunction in FH fibroblasts**

CoQ\textsubscript{10} is an essential component of the mitochondrial electron transport chain (ETC) by transferring reducing equivalents from complexes I and II to complex III \textsuperscript{13}. In order to find out if CoQ\textsubscript{10} deficiency also affects mitochondrial function in FH fibroblasts, we measured the activities of mitochondrial respiratory chain enzymes. Complex I+III activity was significantly reduced in patient compared to control fibroblasts (Fig. 2E). As reduced mitochondrial complexes activity may lead to mitochondrial dysfunction, we next examined mitochondrial membrane potential ($\Delta\Psi_m$) and mitochondrial ROS production. Fig. 2F shows that $\Delta\Psi_m$ was reduced in patient fibroblasts. To asses if cellular bioenergetics was also affected in patient fibroblasts, intracellular ATP levels were also measured. A decrease of 45±13% was found in patient fibroblasts (Fig. 2G). In addition, mitochondrial ROS levels were significantly increased in FH fibroblasts (Fig. 2H).
Selective degradation of mitochondria in FH fibroblasts

As mitochondria dysfunction is often associated with mitophagy activation as a mechanism for the elimination of damaged mitochondria \(^{31,32}\), we next evaluated the amount of acidic vacuoles and the expression levels of autophagy proteins. Acidic vacuoles were examined by Lysotracker red staining coupled to flow cytometry analysis. Higher lysosomal content was found in FH fibroblasts respect to control cells (Fig. 3A).

To asses that high lysosomal activity were accompanied by activation of autophagic processes, mRNA levels and proteins connected to autophagy were examined. The expression of BECLIN, LC3 and ATG5 genes determined by quantitative PCR displayed an increase in FH patients respect to control fibroblasts (Fig. 3B). The same pattern was found when protein levels of ATG5-12 and LC3 were examined by Western blot (Fig. 3C and 3D). Increased levels of Cathepsin B, a lysosomal protein, were also found increased in patient fibroblasts compared to control fibroblasts.

Next, autophagy flux was examined by treating control and FH fibroblast with Bafilomycin, a vacuolar H\(^+\) ATPase inhibitor that prevents the fusion between autophagosomes and lysosomes \(^{33}\), and analyzing LC3-II expression levels by Western blotting. Figure Fig. 3E and 3F show that in control cells bafilomycin treatment increased LC3-II expression levels indicating that autophagy flux was normal. In contrast, the treatment with bafilomycin in FH fibroblasts (P1 and P2) induced only a slight increase in LC3-II expression levels suggesting that autophagy flux was impaired in FH fibroblasts.
To assess the selective elimination of mitochondria in FH cells, protein expression levels of mitochondrial proteins were examined. Expression levels of mitochondrial proteins, VDAC/porine, Complex I (30 kDa subunit) and complex IV (Cox II subunit) were markedly reduced in FH fibroblasts (Fig. 4A). In contrast, protein expression levels of markers of other organelles such as endoplasmic reticulum (PDI), Golgi apparatus (RCAS) and peroxisomes (catalase) were not affected in patient fibroblasts, suggesting that only mitochondria were selectively eliminated in FH fibroblasts (Fig. 4A and 4B). Reduced mitochondrial mass was also confirmed by measuring citrate synthase activity, a well-known marker of mitochondria abundance. Citrate synthase activity was dramatically reduced in patient fibroblasts, indicating extensive mitochondrial loss and/or impaired mitochondrial biogenesis (Fig. 4C).

Mitophagy was also assessed by immunofluorescence microscopy by the examination of co-localization of autophagosome markers as LC3 and mitochondrial markers as cytochrome c. High co-localization between mitochondria and autophagosomes markers (LC3) was found in 80% of analyzed cells in patient fibroblasts (Fig. 5A and 5B).

**Inflammasome activation in FH fibroblasts**

Recent data suggest that damaged mitochondria could induce inflammasome activation \(^34, 35\). It has been described that NLRP3 is able to sense dysfunctional mitochondrialion explaining the common association between mitochondrial damage and inflammatory diseases \(^36\). Inflammation has also
been described as a key process in the formation of the atherosclerotic plaque\textsuperscript{37}.

To assess inflammasome activation in patient fibroblasts, NLRP3 expression levels and Caspase-1 cleavage were analyzed by Western blotting as well as inflammatory cytokines levels, IL-1$\beta$ and IL-18, were measured by ELISA\textsuperscript{38}. Increased expression levels of NLRP3 and caspase-1-cleavage were found in patient fibroblasts (\textbf{Fig. 6A and 6B}) indicating inflammasome activation. Caspase-3 cleavage was also examined to rule out the possibility that inflammasome activation in FH fibroblasts was due to apoptosis activation. Furthermore, IL-1$\beta$ and IL18 levels in culture medium were significantly higher in FH fibroblasts (\textbf{Fig. 6C and 6D}).

\textbf{Effect of CoQ$\textsubscript{10}$ on FH fibroblasts}

As FH fibroblasts showed a dysregulation of mevalonate pathway associated with increased cholesterol and reduced CoQ$_{10}$ biosynthesis, we next examined the effect of CoQ$_{10}$ treatment on cholesterol levels and the expression levels of several key proteins regulating this pathway. P1 and P3 fibroblasts were treated for 72 hours with 25$\mu$M CoQ$_{10}$. Intracellular cholesterol levels were quantified by HPLC (\textbf{Fig. 7A}) and Filipin staining (\textbf{Fig. 7B}). Both determinations showed that cholesterol levels were significantly decreased in CoQ$_{10}$-treated FH fibroblasts, suggesting that CoQ$_{10}$ treatment was able to correct mevalonate pathway. Furthermore, CoQ$_{10}$ treatment reduced significantly cytochrome c/LC3-II puncta (\textbf{Fig. 7C}) indicating improvement of mitochondrial function.
CoQ10 treatment restores altered pathways in FH fibroblasts

In order to explore the molecular mechanism underlying the beneficial effect of CoQ10, the expression levels of several proteins regulating the mevalonate pathways were examined in control and FH fibroblasts. CoQ10 treatment of patient fibroblasts induced AMPK activation and increased PPARα expression levels. This was associated with the restoration of Coq1, Coq2, Coq7 expression levels (Fig. 8 and Supplementary Fig. 4). Furthermore, COQ10 treatment decreased the expression levels of SREBP-2 (mature form), HMGCR and cholesterogenic enzymes such as squalene and lanosterol syntase. In contrast, CoQ10 increased the expression levels of LDL-R and ABCA1 and reduced the expression levels of PCSK9. Interestingly, CoQ10 treatment also increased markedly LDL uptake by FH fibroblasts (Supplementary Fig. 5A and 5B). The beneficial effects of CoQ10 on LDL-R and Coq1 expression levels were dose dependent as is showed in Supplementary Fig. 5C.

To assess the role of AMPK activation in correcting mevalonate pathway under COQ10 treatment we examined the expression levels of essential regulatory proteins of this pathway in the presence or absence of compound C, a selective inhibitor of AMPK. The correction of altered protein expression of enzymes of the mevalonate pathway by CoQ was prevented by AMPK inhibition (Supplementary Fig. 7). Similarly, the effect of CoQ10 on the expression of LDL-R, PCSK9 and NLRP3 was blocked by compound C
(Supplementary Fig. 7). Taking together, these results suggest that the positive effects of CoQ\textsubscript{10} depend on AMPK activation.

**Colesterol accumulation and mitochondrial dysfunction by silencing LDL-R in endothelial cells.**

To reproduce the pathological alterations observed in patient fibroblasts, LDL-R was silenced by siRNA in human endothelial cells. As expected, LDL-R silencing in endothelial cells induced a marked reduction of LDL uptake (Supplementary Fig. 7A and 7B) accompanied by up-regulation of cholesterogenic enzymes such as HMCGR, lanosterol synthase and squalene synthase, and down-regulation of biosynthetic CoQ\textsubscript{10} enzymes such as Coq1 (Supplementary Fig. 7C and 7D). Accordingly, LDL-R silenced cells presented cholesterol accumulation and increased markers of mitochondrial dysfunction and inflammasome activation (Supplementary Fig. 7C-F).
DISCUSSION

In this work, we have examined the pathophysiology of FH in primary cultured fibroblasts derived from 4 patients harboring LDL-R mutations. As a consequence of defective LDL-R, FH fibroblasts showed a marked decrease in the incorporation of cholesterol mediated by LDL-R. Interestingly, intracellular cholesterol levels were considerably high in FH patients, suggesting that cholesterol biosynthesis was up-regulated. Since the seminal works of Brown and Goldstein is well known that defective binding of lipoproteins to cultured fibroblasts induces impaired regulation of HMGCR activity\textsuperscript{39}. This binding appears to be a required step in the process by which LDL normally suppresses the synthesis of HMGCR, the rate-controlling enzyme in cholesterol biosynthesis. The demonstration of a defect in binding of LDL to cells from subjects with the heterozygous form of FH appears to explain the reported failure of lipoproteins to suppress the synthesis of this enzyme and hence may account for the overproduction of cholesterol that occurs in these cultured cells. Thus, increased intracellular lipidogenesis in FH fibroblasts would be a consequence of a constitutive metabolic derepression of enzymes involved in cholesterol synthesis due to defective extracellular cholesterol uptake.

In addition to elevated cholesterol levels and cholesterogenic enzymes expression, a significant decrease in CoQ\textsubscript{10} levels associated with down-regulation of proteins involved in CoQ\textsubscript{10} biosynthesis was also found in FH fibroblasts. These results suggest a possible imbalance in the mevalonate pathway that could affect both the synthesis of cholesterol and CoQ\textsubscript{10}.
In turn, secondary CoQ$_{10}$ deficiency may impair mitochondrial function $^{15}$. Our data confirmed that FH fibroblasts showed mitochondrial dysfunction associated with high ROS production, reduction in ATP levels, diminished mitochondrial membrane potential and low activity of mitochondrial respiratory complexes. These markers of mitochondrial dysfunction have been previously described in several disorders such as fibromyalgia, mitochondrial and lysosomal diseases as well as primary or secondary CoQ$_{10}$ deficiencies $^{19, 32, 40}$.

In addition, autophagy was examined in FH fibroblasts to determine if mutant cells manifested extensive mitophagy as it had been previously described in fibroblasts suffering from mitochondrial dysfunction $^{19, 32, 40}$. Our results showed that autophagic genes and autophagic proteins were increased in FH fibroblasts. Immunofluorescence examination also revealed that mitochondrial markers colocalized with autophagosome markers indicating mitophagy activation. Autophagic flux refers to the whole process of autophagy, including autophagosome formation, maturation, fusion with lysosomes, subsequent breakdown and the release of degraded molecules into the cytosol, representing the dynamic process of autophagy $^{41}$. Our results indicated that autophagy flux was also impaired in FH fibroblasts. This alteration could be responsible for the accumulation of engulfed mitochondria by autophagolysosomes in mutant cells.

Both lipid accumulation and mitochondrial dysfunction have been considered to play an important role for inflammation processes $^{34, 35}$. Indeed, FH fibroblasts showed cholesterol accumulation, mitochondrial dysfunction
and inflammasome activation. Inflammatory processes have been associated with the development of an atherosclerotic plaque, which is created by an oxidative modification of LDL when it is transported to the arterial wall. According to our results, mitochondria dysfunction and CoQ₁₀ deficiency in FH cells can also play an important role in the pathophysiology of early atherosclerosis by contributing to increased production of free radicals and inflammation in the endothelium of blood vessels.

One of the most consistent hypothesis to explain the premature atherosclerosis in FH patients postulates that the disease is due to oxidation of LDL by free radicals produced by circulating and vascular wall cells. However, it is not clear where and how the conditions are set for the generation of oxidative stress in FH. Increased ROS production in isolated mitochondria from liver tissue, cardiac and brain and spleen mononuclear cells isolated from LDL-R knockout mice have been reported. Thus, the defect in LDL-R leads to two important proatherogenic effects: increased levels of oxidized LDL and to an imbalance of cellular redox state. This latter process would be responsible for local stress, which induces lipoprotein oxidation and mitochondrial damage. The resulting vicious cycle would cause cell death and progress of arteriosclerosis in hypercholesterolemia by the absence of LDL-R. This redox imbalance can be important in the pathogenesis of other diseases that also occur with increased hyperlipidemia and lipidogenesis such as diabetes, nephrotic syndrome, obesity, and metabolic syndrome. Within the vascular wall, increased mitochondrial oxidative stress may contribute to the oxidation of
lipoproteins, which, along with increased susceptibility to cell death may be a causal effect on the development of atherosclerotic lesions. In addition, oxidative stress in mitochondria and susceptibility to cell death can contribute to tissue injury of ischemia that occurs in vascular accidents (stroke) and heart attacks in FH.

In our manuscript, we proposed that secondary CoQ10 deficiency due to a constitutive dysregulation of mevalonate pathway may in part explain the generation of oxidative stress in FH cells. Furthermore, mitochondria dysfunction was associated with mitophagy and inflammasome activation, and many experimental studies have reported that NLRP3 inflammasome plays a crucial role in the progression of atherosclerosis. Thus, Duewell and colleagues showed that NLRP3-deficient bone marrow cells transplanted into atherosclerosis-prone low-density lipoprotein receptor–deficient mice had reduced atherosclerosis.

Currently, statins are the main therapeutic option for lowering levels of total cholesterol and LDL cholesterol (LDL-C). Statins act by inhibiting HMGCR. Its development over the past 20 years has represented a breakthrough for the treatment of hypercholesterolemia. Currently, the use of statins is well tolerated and they have a good safety profile. However many adverse effects such as hepatotoxicity, neuropathies, risk of increased incidence of cancer, and various forms of myotoxicity mainly from myalgia to rhabdomyolysis have been described. These deleterious manifestations of muscle occur in some studies in 1-7% of patients treated with statins and do not correlate with the effectiveness on the cholesterol lowering drug used.
The pathophysiological mechanisms of statin-induced myopathy are not fully known. One of the proposed mechanisms postulated that myopathy statins is due to a mitochondrial dysfunction due to inhibition of the mevalonate pathway which is essential for the synthesis of the isoprenoid chain of CoQ10. In turn, CoQ10 is an essential cofactor in the mitochondrial electron transport, and mitochondria are essential to the normal functioning of high energy-demanding tissues such as muscles. Aftermarket studies have indicated that up 13.6% of patients treated with statins experience some degree of myopathy. The diagnosis of the severity of secondary CoQ10 deficiency and mitochondrial impairment in FH patients can be important to prevent potential myotoxic effects caused by treatment with statins at high doses.

For all above reasons, alternative treatments approaches in FH are based on the evidence that statins presumably affects also CoQ10 biosynthesis that is already reduced by an imbalance in the CoQ10/cholesterol biosynthetic pathway in FH. In addition, new therapies are necessary for patients who cannot reach the target LDL-C level when taking the maximum-tolerated dose of a statin or cannot tolerate them. Therefore, treatments focused on reducing intracellular cholesterol content and raising CoQ10 levels would be appropriate for neutralizing mitochondrial damages and restoring the dysregulated choleseterogenic pathway in FH patients.

Sterol regulatory element-binding proteins (SREBPs) are membrane-bound basic helix-loop-helix leucine zipper transcription factors that serve as master regulators of lipid homeostasis by regulating synthesis
of cholesterol, fatty acids, and triglycerides \(^6^0\). The three SREBP isoforms, SREBP-1a, SREBP-1c and SREBP-2, have different roles in lipid synthesis \(^6^1\). In vivo studies using transgenic and knockout mice suggest that SREBP-1c is involved in fatty acid synthesis and insulin induced glucose metabolism (particularly in lipogenesis), whereas SREBP-2 is relatively specific to cholesterol synthesis. When sterol levels decrease by reduce uptake, as it is the case in FH cells, the precursor is cleaved to activate cholesterogenic genes and proteins and maintain cholesterol homeostasis \(^6^2\). Release of membrane-bound SREBP requires SREBP cleavage-activating protein (SCAP) to escort SREBP from the endoplasmic reticulum (ER) to the Golgi for cleavage by site-1 and site-2 proteases. Thus, in FH fibroblasts the low cholesterol uptake provokes increased SREBP-2 maduration and consequently increased cholesterol biosynthesis. However is not clear why increased cholesterol biosynthesis persists despite FH cells contain high cholesterol levels.

Our results clearly showed that both increased cholesterol levels and mitochondrial dysfunction can be restored by treatment with CoQ\(_{10}\), a well-known player in cellular bioenergetics [70]. CoQ\(_{10}\) has been reported to induce AMPK (AMP activated protein kinase) activation \(^6^3\) and peroxisome proliferator-activated receptor \(\alpha\) (PPAR\(\alpha\)) expression via the calcium-mediated AMPK signal pathway \(^6^4\). CoQ\(_{10}\) increases the expression of PPAR\(\alpha\) at both the mRNA and protein levels. Furthermore, knock down of AMPK with siRNA or inhibition of AMPK using an AMPK inhibitor compound C blocked CoQ\(_{10}\)-induced expression of PPAR\(\alpha\), indicating that AMPK plays
a critical role in PPARα induction. On the other hand, AMPK has been reported to inhibit the cleavage and transcriptional activation of SREBP-2, via direct phosphorylation \textsuperscript{65}. Additionally, PPARα can inhibit cholesterol biosynthesis via reduction in SREBP-2 maturation \textsuperscript{66}. In turn, down-regulation of SREBP-2 maturation may reduce the expression levels of HMGCR, squalene synthase and other cholesterogenic proteins.

Our results suggest that CoQ\textsubscript{10} could reduce cholesterol biosynthesis through AMPK-mediated PPARα stimulation. Interestingly, CoQ treatment increases the expression levels of Coq1, Coq2 and Coq7, which have been reported to be under PPARα regulation \textsuperscript{11}. Several studies have reported that AMPK activation reduced lipid synthesis by restraining SREBP activity and led to fatty acid oxidation in the liver to control hepatic energy metabolism \textsuperscript{67}. On the other hand, CoQ\textsubscript{10}-mediated activation of AMPK and/or PPARα may induce LDL-R upregulation as previously it has been reported \textsuperscript{68, 69}. Although, it is known that LDL-R gene promoter activity is enhanced through the proteolytic activation of SREBP-2 and SREBP-1 \textsuperscript{70}, our results showed that LDL-R expression and LDL uptake were markedly increased after CoQ\textsubscript{10} treatment despite reduced maturation of SREBP-2. These findings suggest that LDL-R expression may be regulated by other molecular mechanisms possibly mediated by AMPK and/or PPARα. In agreement with these results, Schmelze et al. have found that treatment with the reduced form of CoQ\textsubscript{10} in humans induces characteristic gene expression patterns, which are translated into reduced LDL-C levels \textsuperscript{71}. Other report suggests that CoQ\textsubscript{10} improves the hypolipemicant action of statins \textsuperscript{72}. CoQ\textsubscript{10} has been intensively
implicated in protecting against chronic diseases, especially atherosclerosis.

It has been proposed that antioxidation and inhibition of inflammation contribute to CoQ10-induced antiatherosclerotic effects. In addition, CoQ10 has been proposed to exert its atheroprotective effects partially by promoting miRNAs-mediated cholesterol efflux by inducing ABCG1 expression. which have been shown to be critically involved in cholesterol and phospholipid efflux in macrophages, hepatocytes, and intestinal mucosa cells. Supporting these results, we found that CoQ10 treatment of FH fibroblasts markedly induced ABCG1 expression.

The therapeutic effect of CoQ10 in cardiovascular diseases is induced by improving of cardiovascular function, preventing LDL oxidation, and inhibiting atherosclerotic processes. There are evidences showing that CoQ10 has an effect on atherosclerotic processes such as improving endothelial function indices, such as increase in the oxygen absorption and flow-mediated dilation and dilated cardiomyopathy. In a study performed by Huynh and co-workers in diabetic patients, CoQ10 decreased oxidative stress, improved lipid profiles, and remodeled left ventricle function. Moreover, it has been shown that oral prescription of CoQ10 can decrease atherosclerotic damages in ApoE-deficient mice.

Conclusion

Our results illustrate that dysregulated mevalonate pathway associated with high intracellular cholesterol content and mitochondrial
dysfunction as well as extensive mitophagy and inflammasome activation may participate in FH pathophysiology (Supplementary Figure 8).

AMPK activation by CoQ$_{10}$ treatment reduced SREBP-2 activation and cholesterol content in FH fibroblasts (Supplementary Figure 9). This finding could be of great interest because AMPK activation has been proposed as a valuable approach to target lipid disorders.

Furthermore, CoQ$_{10}$ treatment improved mitochondrial function and up-regulated LDL-R expression levels in cellular models of the disease. Its underlying mechanism is proposed to be mediated by the activation of AMPK and the concomitant up-regulated expression of PPAR$\alpha$. Therefore, CoQ$_{10}$ can be considered as a promising hypocholesterolemic compound via the normalization of mevalonate pathway in the pharmacotherapy of FH with the benefit of improving mitochondrial function and preventing inflammation.

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Disclosure statement
No potential conflicts of interest were disclosed.
METHODS

Reagents
Monoclonal Anti-Actin antibody, anti-VDAC1/Porin and anti-BECLIN1 were obtained from Sigma- Aldrich (St. Louis, MO). Monoclonal antibodies against complex III (core 1 subunit), complex I (30 kDa subunit) and Complex IV (Cox II subunit), SREBP-2, RCAS (Golgi marker), NRLP3, Mitosox Red, CMH2-DCFDA, 10-N-nonyl acridine orange (NAO), MitoTracker, LysoTracker, tetramethylrhodamine methyl ester (TMRM), MitoTracker Green FM (MTG), Fluorescent LDL Conjugates (Dil LDL) and Hoechst 33342 were obtained from Invitrogen/Molecular Probes (Eugene, OR). Anti-cytochrome c and anti-caspase 3 antibodies were obtained from BD Biosciences Pharmingen (San Jose, CA). Anti-GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) monoclonal antibody (clone 6 C5) was from Calbiochem-Merck Chemicals Ltd. (Nottingham, UK). Anti-hATG12-ATG5 were obtained from Biosensis (South Australia, Australia). Anti-MAP LC3 (N-20), anti-catalase (H-300), anti-PDI (H-160), anti-Golgi marker (AE-6), anti-Cathepsin D, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), anti-LDL-R, anti-caspase 1, anti-AMPK, anti AMPK-p, anti-PPARP-α, anti-SREBP-1, anti-squalene syntase, anti-lanosterol Syntase, anti Coq1, anti Coq2, anti Coq7, anti-PCSK9, anti-ABCA1, LDL-R shRNA and scramble shRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Protease inhibitors were obtained from Boehringer Mannheim (Indianapolis, IN). All other chemicals were purchased from Sigma-Aldrich. Gap, PA, USA).
2-[\textsuperscript{14}C]-(R, S)-mevalonate (55 mCi/mmole) was purchased from Amersham (Buckinghamshire, UK).

**Ethical Statements**

Approval of the ethical committee of the Hospital Universitario Virgen Macarena y Virgen de Rocío de Sevilla (Spain) was obtained, according to the principles of the Declaration of Helsinki and all the International Conferences on Harmonization and Good Clinical Practice Guidelines.

**Fibroblasts cultures**

Cultured fibroblasts were derived from a skin biopsy of patients (P1, P2, P3 and P4) with FH harboring heterozygous null mutations at the LDL-R gene (P1, carries the mutation c.1197_1205delCTACCTCTT; P2 and P3, non-related patients with the same mutation, a deletion from exon 9 to exon 12, c.1187-?_1845+?del; P4, carries the mutation c.12G>A, p.(Trp4*))

Control fibroblasts were human skin primary fibroblasts from healthy volunteers. Samples from patients and controls were obtained according to the Helsinki Declarations of 1964, as revised in 2001. Fibroblasts from FH patients and controls were cultured at 37°C in DMEM containing 4.5 g·L\textsuperscript{−1} glucose, L-glutamine, and pyruvate supplemented with 1% antibiotic solution and 20% Fetal Bovine Serum (FBS).

**LDL-R silencing in endothelial cells**

Endothelial cells (EA.hy926) were a generous donation from Carmelo Bernabeu Quirante (Centro Investigaciones Biológicas, CIB). Cells were seeded in two 12-wells plates, silenced control and silenced LDL-R in DMEM medium with 4.5 g/l glucose, 20% fetal bovine serum and 1% antibiotics
(Optimal medium). After reaching a confluence of 50%, cells were washed once with PBS and replaced with optimal medium plus 10 μg/ml of Polybrene (Santa Cruz Biotech). shRNA Lentiviral particles (Santa Cruz Biotech) were added to the culture (shControl/shLDL-R) and incubated overnight. Cell were washed with PBS once and replaced with optimal medium and incubated overnight. Every well were split in 3 different T25 Flasks and continue incubating for 48 hours in optimal medium. To select transfected and stable clones, Puromycin (Santa Cruz Biotech) was added in a concentration of 2 μg/ml. Puromycin and medium was refreshed every week.

**LDL uptake**

Cultured fibroblasts were incubated 2 hours at 37°C with culture medium mixed with human fluorescent LDL complexes (Molecular Probes, Eugene, OR, USA) at 12 μg/mL. After incubation, cells were rinsed twice with culture medium and visualized *in vivo* using an upright fluorescence microscope (Leica DMRE, Leica Microsystems GmbH, Wetzlar, Germany).

**Oil red-O staining**

Fibroblasts were grown on 1 mm width glass coverslips for 72 h in culture medium. Cells were rinsed twice with PBS, fixed in 3.8% paraformaldehyde for 5 min at room temperature, and permeabilized with 0.1% saponin for 5 min. A 6:10 dilution was made by a 5% Oil red solution prepared in 2-propanol and added to cells for 60 minutes at room temperature. The staining solution was removed and cells were washed with PBS three times before viewing by optical microscopy.
Filipin staining

Fibroblasts were grown on 1 mm width glass coverslips for 72 h in normal growth medium. Cells were rinsed twice with PBS, fixed in 3.8% paraformaldehyde for 5 min at room temperature and rinsed again. Cells were incubated for 10 min at room temperature with 1.5 mg glycine/ml PBS. A working solution with 0.05 mg/mL in PBS was prepared from a SIGMA F4767 stock diluted in DMSO and added to fibroblasts for 2 hours at room temperature. Cells were rinsed twice with PBS and visualized by fluorescence microscopy using a UV filter set.

Measurement of cholesterol and CoQ\textsubscript{10} levels

Fibroblast pellets were lysed with 500µL PBS 1x and mixed vigorously. Then, 20 µL of 30 µM Coenzyme Q\textsubscript{9} was added as internal standard. 500µL SDS 2% and 2mL of Ethanol:isopropanol (95:5) were added to each sample and mixed. To extract lipids, 4 ml of hexane were added and vortexed. Samples were centrifuged at 1000×g for 5 min at 4 °C and the upper phase was recovered. This step was repeated twice and 12mL were dried via rotary evaporation. Lipid extracts were vortexed with 900µL of ethanol (HPLC quality) and dried in a speed-vac. The lipid pellet was suspended and homogenized in 100µL of ethanol prior to HPLC injection. Lipid components were separated by a SHIMADZU UFLC HPLC system equipped with a reversed-phase Shim-pack XR-ODS C-8 column in a column oven set to 40 °C, with a flow rate of 0.5 ml/min and a mobile phase containing 80:20 methanol/2-propanol. CoQ\textsubscript{9}, CoQ\textsubscript{10} and Cholesterol levels
were analyzed with a SPD-20A prominence UV-VIS detector at 200 and 245nm, respectively.

**De novo synthesis of cholesterol and CoQ<sub>10</sub>**

Radiative mevalonate was added to fibroblast cultures at 10,000 dpm/mL and incubated for 48 hours at 37°C and 5% CO<sub>2</sub>. Lipid extraction was developed as indicated previously and samples were analyzed by thin layer chromatography and a 2D radioactivity detector.

**Immunofluorescence microscopy**

Fibroblasts were grown on 1 mm width glass coverslips for 72 h in normal growth medium. Cells were rinsed twice with PBS, fixed in 3.8% paraformaldehyde for 5 min at room temperature, and permeabilized with 0.1% saponin for 5 min. Glass coverslips were incubated at 37°C with primary antibodies diluted 1:100 in PBT for an hour and rinsed twice with PBS. The secondary antibody, diluted 1:100 in PBS, was incubated for 45 minutes at 37°C. The coverslips were then rinsed twice with PBS, incubated for 4 minutes with PBS containing Hoechst 33342 dilution 1:1000 and washed with PBS. The coverslips were mounted onto microscope slides using Vectashield Mounting Medium and analyzed using an upright fluorescence microscope (Leica DMRE, Leica Microsystems GmbH, Wetzlar, Germany). Images were taken using a DeltaVision system (Applied Precision; Issaquah, WA, USA) with an Olympus IX-71 microscope using a 100× objective.
**Immunoblotting**

Western blotting was performed using standard methods. After transferring the proteins, the membranes were incubated with primary antibodies at 1:1000, overnight, rinsed twice and incubated again with the corresponding secondary antibody.

**ATP levels**

An ATP determination kit (Invitrogen-Molecular Probes) was used to measure ATP levels by a bioluminescence assay.

**Mitochondrial membrane potential ($\Delta\Psi_m$)**

FH fibroblasts were grown on multiwell plates for 24 to 48 hours, incubated with 100 nM TMRM for 30 minutes and examined by flow cytometry.

**Mitochondrial mass**

Mitochondrial mass was determined by flow cytometry and fluorescence microscopy after staining of cells with 10 µM 10-$N$-nonyl acridine orange (NAO) for 10 min at 37°C in the dark.

**Mitochondrial reactive oxygen species (ROS) generation**

Mitochondrial ROS generation in fibroblasts was assessed by MitoSOX, a red mitochondrial superoxide indicator. Once in the mitochondria, MitoSOX red reagent is oxidized by superoxide and exhibits red fluorescence. Approximately $1 \times 10^6$ cells were incubated with 1 µM MitoSox.
for 30 min at 37°C, washed twice with PBS, resuspended in 500 μl of PBS, and analyzed by flow cytometry (excitation at 510 nm and fluorescence detection at 580 nm). Specificity of MitoSOX for superoxide has been shown by the manufacturer, and its mitochondrial localization was tested by costaining with MitoTracker Green (data not shown). ROS levels were expressed relative to the mitochondrial mass (ROS signal/NAO signal).

**Mitochondrial respiratory chain activity**

Activities of NADH: cytochrome c reductase (complexes I+III) and citrate synthase were determined spectrophotometrically in sonicated and permeabilized fibroblasts using previously described methods. Results are expressed as units/citrate synthase (mean±SD). Protein content was determined by the Lowry procedure.

**Real-time quantitative PCR of autophagic genes**

The fibroblast expression of ATG12, MAP-LC3, BECLIN, COQ1, COQ2, SREBP-2, HMGCR and LSS (lanosterol synthase) genes in fibroblasts was analyzed by SYBR Green quantitative PCR using mRNA extracts and primers. Real-time BECLIN1 primers 5'-GGATGGATGTGGAGAAAGGCAAG-3' (forward) and 5'-TGAGGACACCCAAGCAAGACC-3' (reverse) amplify a sequence of 152 nt. Human ATG12 primers 5'-ATTGCTGCTGGAGGGGAAGG-3' (forward) and 5'-GGTTCGTGTTGCTCTACTGC-3' (reverse) amplify a sequence of 198 nt. Human MAP-LC3 primers 5'-GCCTTCTTCCTGCTGGTAAC-3' (forward)
and 5-AGCCGTCTCGTCTTTCTCC-3 (reverse) amplify a sequence of 91 nt. Human COQ1 primers 5-TTCAACAGCGACACCCACT-3 (forward) and 5-CTGCAATGGACTAGCTCTGC-3 (reverse) amplify a sequence of 179 nt. Human COQ2 primers 5-GCGAGAGCGTTGACTTAAG-3 (forward) and 5-GCGTTACTGGATGGTCTGC-3 (reverse) amplify a sequence of 160 nt. Human SREBP-2 primers 5-GGAGTGGTGCTGAATGTGG-3 (forward) and 5-TTTCTCCCACCTCAGTCCC-3 (reverse) amplify a sequence of 132 nt. Human HMGCR primers 5-CACAACAGCTCCCATCACC-3 (forward) and 5-GGAAACTCATGAGCGTGTTG-3 (reverse) amplify a sequence of 127 nt. Human LSS primers 5-CCCAACACAGTTCTTCAGGC-3 (forward) and 5-TCTTCTGTCCAGCTCCCTTG-3 (reverse) amplify a sequence of 163 nt. GADPH was used as control gene.

**Electron microscopy**

Electron microscopy was performed as described 19. Culture cells were fixed with 2% glutaraldehyde for 15 minutes at RT and for 30 minutes in 2% glutaraldehyde 0.1M NaCacodylate/HCl at pH 7.4. Fibroblasts were washed with 0.2 M NaCacodylate/ HCl, pH 7.4 and fixed again for 30 minutes 1% OsO4- 0.15 M NaCacodylate/HCl, pH 7.4. Samples were dehydrated with ethanol at 30, 50, 70 and 95% for 5 minutes. Impregnation and inclusion steps were performed in Epon and polymerized for 48h at 60°C. An ultramicrotome RMC-MTX (Tucson, Arizona) was used to get 60-80nm sections. Sections were contrasted with uranyl acetate and lead citrate.
Philips CM-10 transmission electron microscope was used to capture images.

**Lysosomal content assay**

Fibroblasts were cultured in multiwell plates and incubated for 30 minutes at 37°C with 100nM Lisotracker Red (Molecular Probes). After incubation, cells were washed and the red fluorescence of LysoTracker™ was measured by flow cytometry.

**Interleukinas**

IL-1β and IL-18 levels in culture mediums were assayed by commercial ELISA kits (MyBioSource, Inc., CA, USA).

**Statistical analysis**

All results are expressed as mean±SD of 3 independent experiments. The measurements were statistically analyzed using the Student’s t test for comparing 2 groups and analysis of variance for more than 2 groups. The level of significance was set at p<0.05.

**Abbreviations:**

ABCA1, ATP-binding cassette sub-family A member 1; ATG5/12, autophagy related 5/12; BECN1 beclin 1, autophagy related; COQ10, coenzyme Q_{10}; DNM1L, dynamin 1-like; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; GADPH, glyceraldehyde-3-phosphate
dehydrogenase; HMGCR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; INSIG-1, insulin induced gene 1; MAP1LC3A/B, microtubule-associated protein 1 light chain 3 alpha/beta; LDLRAP1, LDL receptor adaptor protein 1; LDL-R, low density lipoprotein receptor; LXR, liver X receptor; 3MA, 3-methyladenine; MFN1, mitofusin; MFN2, mitofusin 2; mtDNA, mitochondrial DNA; MTOR, mechanistic target of rapamycin (serine/threonine kinase); NRLP3, nacht Domain-, leucine-rich repeat-, and PYD-containing protein 3; PDI, protein disulfide isomerase; PCSK9, proprotein convertase subtilisin/kexin type 9; PPAR-α, peroxisome proliferative activated receptor alpha; RCAS, receptor binding cancer antigen expressed on SiSo cells; SDHA, succinate dehydrogenase complex, subunit A, flavoprotein (Fp); SREBP, sterol regulatory element binding proteins; TFAM, transcription factor A, mitochondrial; TMRM, tetramethylrhodamine methyl ester; VDAC1, voltage-dependent anion channel 1.
REFERENCES

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Figure legends

Figure 1. Impaired LDL uptake and cholesterol accumulation in FH fibroblasts. (A) Control and FH fibroblasts were incubated with a fluorescently-labeled LDL and examined by fluorescence microscopy as described in Material and Methods. An impaired LDL-uptake was found in FH fibroblasts. Bar =15 μm. (B) Quantification LDL-uptake by Image-J software. (C) LDL-R expression. Expression levels of LDL-R in control and FH fibroblasts determined by Western blotting. For control cells, data are a pool of 2 different control cell lines. (D) Red oil staining in control and FH fibroblasts was performed as described in Materials and Methods. Bar =15 μm. (E) Image analysis of red oil staining. (F) Electron Microscopy in control and FH fibroblasts was performed as described in Materials and Methods. Bar =1 μm (G) Filipin staining in control and FH fibroblasts was performed as described in Materials and Methods. Cells were then examined under fluorescence microscopy. Bar =15 μm. (H) Filipin quantification by Image J software. Data represent the mean±SD of 3 separate experiments. *p<0.05 between control and FH fibroblasts. **p<0.01 between control and FH fibroblasts. A.U., arbitrary units.

Figure 2. Dysregulated mevalonate pathway and mitochondrial dysfunction in FH fibroblasts. Cholesterol (A) and CoQ (B) biosynthesis analysis in control and FH fibroblasts was performed incubating cells with radioactive mevalonate as described in Materials and Methods. For control cells, the data are the mean±SD for experiments conducted on 2 different
control cell lines. (C) Cholesterol levels in control and FH fibroblasts were determined by hexane extraction and HPLC separation as described in Materials and Methods. (D) CoQ levels in control and FH fibroblasts were determined by hexane extraction and HPLC separation as described in Materials and Methods. (E) Mitochondrial enzymatic activities of complex I+III in control and FH fibroblasts was determined as described in Materials and Methods. (F) Mitochondrial membrane potential (ΔΨm) was assessed by flow cytometry using MitoTracker Red. A clear decrease of ΔΨm was observed in FH fibroblasts. (G) ATP levels in control and FH fibroblasts. A significant decrease of ATP levels was observed in FH fibroblasts. (H) Mitochondrial ROS levels in control and FH fibroblasts. Results are expressed as the ratio of MitoSOX signal to 10-N-nonyl acridine orange signal. For control cells, the data are the mean±SD for experiments conducted on 3 different control cell lines. Data represent the mean±SD of 3 separate experiments. *p<0.05 between control and FH fibroblasts. **p<0.01 between control and FH fibroblasts. A.U., arbitrary units.

Figure 3. Increased expression of autophagic markers in FH fibroblasts. (A) Quantification of acidic vacuoles in control and FH fibroblasts by LysoTracker staining and flow cytometry analysis as described in Material and Methods. (B) Increased expression of autophagic transcripts. Expression levels of BECLIN, LC3 and ATG5 were performed by RT-PCR as described in Material and Methods. (C) Increased expression of autophagic proteins. The expression levels of LC3-I (upper band) and LC3-II (lower band),
ATG12, BECLIN1 and cathepsin D were determined in the control and FH fibroblast cultures by Western blotting. The ATG12 band represents the Atg12-Atg5 conjugated form. Actin was used as a loading control. (D) The densitometric analysis of Western blottings. (E) Autophagy flux in FH fibroblasts. Determination of LC3-II levels in the presence and absence of bafilomycin A1 in control and FH fibroblasts. Control and FH fibroblasts were incubated with bafilomycin A1 (100 nM for 12 h). Total cellular extracts were analyzed by immunoblotting with antibodies against LC3. Actin was used as a loading control. (F) Densitometry of Western blotting was performed using the ImageJ software. For control cells, the data are the mean±SD for experiments conducted on 3 different control cell lines. Data represent the mean±SD of 3 separate experiments. *p<0.01 between control and FH fibroblasts. A.U., arbitrary units.

Figure 4. Mitophagy in FH fibroblasts. (A) Western blot analysis of mitochondrial (complex I, 30 kDa subunit; complex IV, COX II subunit; and porin), Golgi (Golgi marker), endoplasmic reticulum (PDI), and peroxisome (catalase) proteins in control and FH fibroblasts. Actin was used as loading control. (B) Densitometry of Western blotting. (C) Mitochondrial mass was determined by determining citrate synthase activity as described in Material Methods. For control cells, the data are the mean±SD for experiments conducted on 3 different control cell lines. Data represent the mean±SD of 3 separate experiments. *p<0.01 between control and FH fibroblasts. A.U., arbitrary units.
Figure 5. Autophagosome and mitochondria markers colocalization in FH fibroblasts. (A) Image analysis of LC3 and cytochrome c immunostaining in control and FH fibroblasts. Control and FH fibroblasts were fixed and immunostained with anti-LC3 (autophagosome marker) and cytochrome c (mitochondrial marker) and examined by fluorescence microscopy. Nuclei were revealed by Hoechst staining. Scale bar=15 μm (B) Magnification of a small area in a FH fibroblast. Yellow arrow shows autophagosomes with LC3 and cytochrome c colocalization. Red arrow shows tubular mitochondria without colocalization with LC3. Colocalization of both markers was assessed using the DeltaVision software and calculating the Pearson’s coefficient of correlation. Scale bar=5 μm. (C) Quantification of LC3/cytochrome c punctata in control and FH (n=100 cells). Data represent the mean±SD of 3 separate experiments. *p<0.05 between control and FH fibroblasts. **p<0.01 between control and FH fibroblasts.

Figure 6, Inflammasome activation in FH fibroblasts. (A) Western blot analysis of NLRP3, caspase-1 and IL-1β in control and FH fibroblasts. (B) Densitometric analysis of Western blottings. Data represent the mean±SD of three separate experiments. *p<0.05 between control and FH fibroblasts. a*p<0.05 between the presence and the absence of CoQ treatment. (C) IL-1β and IL-18 levels were determined by ELISA assay as described in Material and Methods. Data represent the mean±SD of three separate experiments. *p<0.01 between control and FH fibroblasts. A.U., arbitrary units.
Figure 7. CoQ treatment restores cholesterol levels and reduces mitophagy in FH fibroblasts. (A) Control and FH fibroblasts were treated with 25 µM CoQ₁₀ for 72 hours. Cholesterol levels in control and FH fibroblasts were determined by hexane extraction and HPLC separation as described in Materials and Methods. (B) Filipin staining in control and FH fibroblasts treated with CoQ. (C) Quantification of puncta (LC3 and cytochrome c colocalization) in control and FH fibroblasts treated with CoQ. Control and FH fibroblasts were fixed and immunostained with anti-LC3 (autophagosome marker) and cytochrome c (mitochondrial marker) and examined by fluorescence microscopy. For control cells, the data are the mean±SD for experiments conducted on 2 different control cell lines. Data represent the mean±SD of 3 separate experiments. *p<0.05 between control and FH fibroblasts. #p<0.01 between the presence and the absence of CoQ treatment.

Figure 8. Effect of CoQ₁₀ treatment on several pathways regulating mevalonate pathway. (A) Western blot analysis of SREBP-2 processing, AMPK activation, and expression levels of PPAR-α, HMGCR, lanosterol synthase, squalene synthase, Coq2, Coq7, NLRP3, PCSK9, ABCG1 and LDL-R. Control and FH fibroblasts were treated with 25 µM CoQ₁₀ for 72 hours. Actin was used as loading control.
Figure 2
Figure 3
Figure 4
Figure 5

A

Cyt C | LC3B | HOESCHT | MERGE

P1 | P2 | P3 | P4

B

Puncta LC3/Cyt C

Control | P1 | P2 | P3 | P4

Figure 5
**Figure 6**

**A**  
INFLAMMASOME

![Image of Western Blot](image)

**B**

![Graph showing band intensity (A.U.)](image)

**C**

![Graph showing IL-1b (pg/ml)](image)

**D**

![Graph showing IL-18 (pg/ml)](image)
Figure 7

Panel A: Bar chart showing pmol cholesterol/mg protein. P1 and P3 treatments with CoQ10 show significant increases compared to controls.

Panel B: Images showing punctae LC3/Cyt C staining. CoQ10 treatment reduces punctae in P1 and P3 conditions.

Panel C: Bar chart showing punctae LC3/Cyt C. P1 and P3 treatments with CoQ10 show significant increases compared to controls.
Figure 8
Supplementary Material

**Supplementary Figure 1.** Immuno fluorescence of LDL-R. Control and FH fibroblasts were fixed and immunostained with anti-LDL-R and examined by fluorescence microscopy. Nuclei were revealed by Hoechst staining. Scale bar=15 μm.

**Supplementary Figure 2.** Expression levels of proteins involved in cholesterol (SERB-2, HMGCR, lanosterol and squalene synthase) and CoQ₁₀ (Coq1, Coq2 and Coq7) biosynthesis in control and FH fibroblasts were analyzed by Western blotting.

**Supplementary Figure 3.** (A) and (B) Densitometry of Western blotting of Supplementary Figure 2. (C) Expression levels of cholesterogenic and CoQ₁₀ biosynthetic transcripts. Expression levels of HMCGR, lanosterol synthase, SREBP-2, COQ1 and COQ2 were performed by RT-PCR as described in Material and Methods. Data represent the mean±SD of 3 separate experiments. *p<0.01 between control and FH fibroblasts. A.U., arbitrary units.

**Supplementary Figure 4.** Densitometric analysis of Western blotting of Figure 8. Data represent the mean±SD of three separate experiments. *p<0.01 between control and FH fibroblasts. #p<0.01 between the presence and the absence of CoQ treatment. A.U., arbitrary units.

**Supplementary Figure 5. Effect of CoQ₁₀ treatment on FH fibroblasts.** (A) Effect of CoQ₁₀ on LDL uptake. After 25 μM CoQ₁₀ treatment for 72 hours, control and FH fibroblasts were incubated with a fluorescently-labeled LDL and examined by fluorescence microscopy as described in Material and Methods. CoQ₁₀ treatment improves the impaired LDL-uptake in FH fibroblasts (B)
Quantification LDL-uptake by Image-J software. (C) Western blot analysis of LDL-R and Coq1 expression levels under increasing concentrations of CoQ_{10} (1-50 μM) in patient fibroblast (P1). Data represent the mean±SD of three separate experiments. *p<0.05 between control and FH fibroblasts. °p<0.05 between the presence and the absence of CoQ_{10} treatment. A.U., arbitrary units.

**Supplementary Figure 6. Effect of AMPK inhibition by compound C on CoQ_{10} treatment.** Control and FH fibroblasts were treated with 25 μM CoQ_{10} in the presence of 10 μM compound C (CC), an AMPK inhibitor, for 72 hours (A) Western blot analysis of AMPK, PPAR-α, Coq2, Coq7, lanosterol synthase, PKSK9 and NLRP3. (B) Desitometry of Western blotting. *p<0.05 between control and FH fibroblasts. °p<0.01 between the presence and the absence of CoQ_{10} treatment. A.U., arbitrary units.

**Supplementary Figure 7. Effects of LDL-R silencing on human endothelial cells.** (A) Control, control siRNA (SCRm), and LDL-R silenced endothelial cells were incubated with a fluorescently-labeled LDL and examined by fluorescence microscopy as described in Material and Methods. An impaired LDL-uptake was found in LDL-R silenced endothelial cells. (B) Quantification LDL-uptake by Image-J software. (C) Western blot analysis of LDL-R, HMGCR, squalene synthase, lanosterol synthase, Coq1 and NLRP3 in cell extracts from control, control siRNA, (SCRm) and LDL-R silenced endothelial cells. Actin was used as loading control. (D) Densitometry of Western blotting (E) Quantification of cholesterol in control, control siRNA (SCRm), and LDL-R silenced endothelial cells by Filipin staining. (F) Mitochondrial dysfunction addressed by measuring mitochondrial membrane potential (ΔΨm) by flow cytometry as described in
Material and Methods. *p<0.01 between control and silenced cells. A.U., arbitrary units.

**Supplementary Figure 8. Working model of FH physiopathology.** Low expression of LDL-R results in dysregulated mevalonate pathway which leads to cholesterol accumulation and secondary CoQ_{10} deficiency. As a consequence, mitochondrial dysfunction produces oxidative stress and mitophagy and inflammasome activation.

**Supplementary Figure 9. Working model of the impact of CoQ_{10} treatment in FH physiopathology.** CoQ_{10} treatment can correct both altered mevalonate pathway and mitochondrial function in FH fibroblasts. CoQ_{10} also causes a significant increase expression of LDL-R and ABCA1 accompanied by downregulation of PCSK9. As a consequence intracellular cholesterol levels are restored to control values.
Supplementary Figure 1

A

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Supplementary Figure 2
Supplementary Figure 3
Supplementary Figure 4
Supplementary Figure 6
Supplementary Figure 7
HIPOTHESES HF

- Mitochondrial dysfunction
- Oxidative stress
- Mitophagy
- Impaired autophagy flux
- Inflammasome

LDL-R \(\downarrow\) → Low cholesterol uptake \(\rightarrow\) SREBP-2

PCSK9 \(\uparrow\)

ABCA1 \(\downarrow\)

Coq 7 \(\downarrow\)

decaprenyl-4-hydroxybenzoate

coenzyme Q_{10}

Coq 2

decaprenyl-PP

decaprenyl

FPP

2,3-oxidosqualene

HMG-CoA reductase

HMG-CoA

mevalonate

acetyl-CoA

Supplementary Figure 8
HIPOTHESES HF+CoQ<sub>10</sub>

CoQ<sub>10</sub> → AMPK/PPARα → SREBP-2

LDL-R → Normal cholesterol uptake

PCSK9

ABCA1

↑ Coq 1 → decaprenyl-PP

↑ Coq 2

↑ Coq 7 → decaprenyl-4-hydroxybenzoate → coenzyme Q<sub>10</sub>

HMG-CoA reductase → mevalonate → FPP → 2,3-oxidosqualene → lanosterol → cholesterol =

Improvement of Mitochondrial function

Supplementary Figure 9