Unveiling the Mechanisms for Statin-Associated Sleep Problems and Myopathy

Statin Medication, Sleep Problems and Myopathy Mechanisms

MOHAMED H. AL-SABRI
Dissertation presented at Uppsala University to be publicly examined in room A1:111a, BMC, Husargatan 3, Uppsala, Wednesday, 5 June 2024 at 06:36 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English. Faculty examiner: Associate Professor Sabata Pierno (Dept. of Pharmacy & Drug Sciences, University of Bari).

Abstract

Statins (3-hydroxy-3-methylglutaryl-CoA reductase, HMGCR, inhibitors) comprise the gold standard for the management of hypercholesterolaemia and prevention of cardiovascular disease (CVDs). However, they are accompanied by potential adverse effects, notably muscle pain and sleep disturbance. These side effects can significantly impact patient adherence to statin therapy and thus increase the risk for CVDs. Despite extensive research, the underlying mechanisms of statin-associated myopathy and sleep disturbance are poorly understood.

In Paper I, we conducted a cross-sectional cohort study to investigate the association between statin use and genetic variants for HMGCR with the risk for insomnia and chronotype using UK biobank cohort data. Statin use, insomnia and chronotype were assessed by a self-report touchscreen questionnaire. Statin treatment was associated with an increased risk of insomnia compared to controls, while genetic variants for HMGCR inhibition were associated with a reduced risk for insomnia. No association with late evening chronotype were observed with statin use or genetic variants for HMGCR.

In Paper II, we employed Drosophila melanogaster to examine the effect of statins and the role of central inhibition of Hmgcr on sleep behaviour. Flies were treated with fluvastatin for five days and Hmgcr was knocked down in pan neurons and pars intercerebralis (PI), equivalent to the mammalian hypothalamus. Sleep patterns were recorded and analysed. Pan-neuronal- as well as PI inhibition of Hmgcr recapitulates fluvastatin-induced enhanced sleep latency and reduced sleep duration.

In Paper III, we deciphered the underlying mechanisms for statin-induced myopathy using D. melanogaster. We found that fluvastatin treatment induced muscular damage, mitochondrial phenotypes, lowered locomotion, reduced climbing activity and was associated with lipotoxicity, impaired muscle differentiation and regeneration, and lowered expression of skeletal muscle chloride channels. Interestingly, selective inhibition of skeletal muscle chloride channels recapitulates fluvastatin-induced myofibrillar damage and lowered climbing activity, while selective Hmgcr inhibition in the skeletal muscles recapitulates fluvastatin-induced mitochondrial round-shape and reduced locomotion activity.

In Paper IV, we explored the sequential events of myofibril damage and mitochondrial phenotypes associated with fluvastatin and examined whether inhibition of Hmgcr in the skeletal muscles recapitulates fluvastatin effects on mitochondrial respiratory parameters using D. melanogaster. Acute fluvastatin treatment was associated with reduced mitochondrial content and roundness of the mitochondria without noticeable myofibrillar damage. Intriguingly, chronic fluvastatin treatment was associated with stronger mitochondrial phenotypes along with severe myofibrillar damage, which suggests that mitochondrial phenotypes precede myofibrillar damage. Moreover, selective Hmgcr inhibition did not impact mitochondrial respiratory functions.

Mohamed H. Al-Sabri, Department of Surgical Sciences, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden.

© Mohamed H. Al-Sabri 2024

ISSN 1651-6206
URN urn:nbn:se:uu:diva-525998 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-525998)
To whom Taught me the first letter: Father & Mother
To whom supported me relentlessly: My Wife and Children
This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


IV. **Mohamed H. Al-Sabri**, Nourhane Ammar, Stanislava Korzh, Ahmed M. Alsehli, Kimia Hosseine, Robert Fredriksson, Jessica Mwinyi, Michael J. Williams, Hadi Boukhatmi, Helgi B. Schiöth 2024. Fluvastatin-Induced Myofibrillar Damage Is Associated with Elevated ROS, and Impaired Fatty Acid Oxidation, and is Preceded by Mitochondrial Morphological Changes. Accepted to Scientific Reports. Doi.org/10.1038/s41598-024-53446-w

Reprints were made with permission from the respective publishers.
List of Papers not included in the Thesis


Contents

Introduction ........................................................................................................... 9
  Statin therapy .................................................................................................. 9
  Discovery of Statins and Mechanism of Action ............................................. 9
  Statin intolerance ............................................................................................ 10

Aims ...................................................................................................................... 12
  Paper I ............................................................................................................ 12
  Paper II ............................................................................................................ 12
  Paper III .......................................................................................................... 12
  Paper IV ........................................................................................................... 13

Materials and Methods ...................................................................................... 14
  *Drosophila* work .......................................................................................... 14
    Functional studies: Gene manipulation ....................................................... 14
    Fluvastatin concentration .......................................................................... 15
    Quantification of locomotion and climbing activities ................................. 16
    Immunohistochemistry and morphology examination .................................. 16
    Quantification of gene expression ............................................................... 16
    Mitochondrial respiration ........................................................................... 17

Cross-sectional observation study ..................................................................... 18
  Data set ............................................................................................................ 18
  Genetic analysis .............................................................................................. 18
  Statin treatment and sleep patterns ............................................................... 18
  Statistical Analysis ......................................................................................... 19

Results .................................................................................................................. 20
  Paper I ............................................................................................................ 20
  Paper II ............................................................................................................ 21
  Paper III .......................................................................................................... 21
  Paper IV ........................................................................................................... 24

Discussions and conclusions ............................................................................. 26

Perspectives ........................................................................................................... 29

Acknowledgement .............................................................................................. 31

References .......................................................................................................... 33
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>HMGR</td>
<td>3-hydroxy-3-methylglutaryl-coenzyme A reductase</td>
</tr>
<tr>
<td>PCSK9</td>
<td>Proprotein convertase subtilisin/kexin type 9</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>ACC / AHA</td>
<td>American College of Cardiology and American Heart Association</td>
</tr>
<tr>
<td>LDL-C</td>
<td>Low-density lipoprotein cholesterol</td>
</tr>
<tr>
<td>Lpin</td>
<td>Lupin</td>
</tr>
<tr>
<td>Dgat2</td>
<td>Diacylglycerol O-acyltransferase 2</td>
</tr>
<tr>
<td>Hnf4</td>
<td>Hepatocyte nuclear factor 4-gamma</td>
</tr>
<tr>
<td>Mef2</td>
<td>Myocyte enhancer factor-2</td>
</tr>
<tr>
<td>Mhc</td>
<td>Myosin heavy chain</td>
</tr>
<tr>
<td>Zfh1</td>
<td>Zn finger homeodomain 1</td>
</tr>
<tr>
<td>FAO</td>
<td>Fatty acid oxidation</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single-nucleotide polymorphisms</td>
</tr>
<tr>
<td>DAMS</td>
<td>Drosophila activity monitoring system</td>
</tr>
<tr>
<td>CPT2</td>
<td>Carnitine palmitoyltransferase 2</td>
</tr>
<tr>
<td>IPCs</td>
<td>Insulin-producing cells</td>
</tr>
<tr>
<td>PI</td>
<td>Pars intercerebralis</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglyceride</td>
</tr>
<tr>
<td>PPARα</td>
<td>Peroxisome proliferator-activated receptor alpha</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase c</td>
</tr>
<tr>
<td>FKBP12</td>
<td>Fk506 binding protein</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>SIM</td>
<td>Statin induced myopathy</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
</tbody>
</table>
Introduction

Statin therapy

For decades, cardiovascular diseases (CVDs) have consistently ranked as the first leading causes of morbidity and mortality worldwide, accounting for 20.5 million deaths in 2021 alone\textsuperscript{1,2}. CVDs encompass a wide range of disorders including coronary heart and cerebrovascular diseases such as ischaemic heart disease and atherosclerosis, with hypercholesteremia as the primary risk factor\textsuperscript{1,2}. Over time, elevated plasma cholesterol levels lead to arterial plaque formation, resulting in arterial hardening, reduced blood flow, and activation of the clotting cascade—a process known as atherothrombosis\textsuperscript{3}.

Statins (HMGCR inhibitors) reduce blood cholesterol by inhibiting the rate-limiting step in intracellular cholesterol biosynthesis and thus thrombogenicity\textsuperscript{4,5}. Statins are among the top-ten prescribed drugs worldwide with 25\% of adults over 65 years currently under statin treatment, and this percentage is estimated to increase statin users in the future\textsuperscript{6}. According to recent guidelines from the American Heart Association (AHA) for the management of hypercholesterolemia, the number of adults who would be eligible for statin therapy is set to climb from 43.2 million (37.5\%) to 56.0 million (48.6\%), owing to the inclusion of older adults pre-existing CVDs\textsuperscript{7}.

Discovery of Statins and Mechanism of Action

The mevalonate pathway is a highly conserved pathway responsible for the intracellular biosynthesis of cholesterol and isoprenoids, where HMGCR functions as the rate-limiting enzyme\textsuperscript{8}. The full delineation of the cholesterol biosynthesis pathway by Bloch and Lynen in 1960 was a big milestone towards the discovery of Statins in 1976\textsuperscript{9}. In 1985, lovastatin was the first commercially launched statin by Merck\textsuperscript{9}. Statins bind and competitively inhibit HMGCR with an affinity to HMGCR that is 10,000 times higher than the substrate (HMG-CoA)\textsuperscript{4}, and therefore reduce the biosynthesis of cholesterol. Statins increase the clearance of cholesterol by two connected mechanisms. First, statins inhibit intracellular biosynthesis of cholesterol by inhibiting HMGCR in many tissues but mainly in the liver. This leads to enhanced expression of LDL receptors in hepatocytes, resulting in uptake of LDL
cholesterol (LDL-C), also referred to as 'bad cholesterol', from the blood and ultimately decreased plasma levels of LDL-C\textsuperscript{10,11} (Fig. 1).

![Diagram showing statin mechanisms for reduction of blood cholesterol levels. 1. Statins inhibit HMGCR, the rate-limiting step of hepatocyte cholesterol biosynthesis. 2. Inhibition of cholesterogenesis leads to enhanced expression of LDL receptors in hepatocytes, resulting in increased uptake of LDL-C (bad cholesterol) from the blood and ultimately reduced plasma cholesterol.]

Fig. 1. Statin mechanisms for reduction of blood cholesterol levels. 1. Statins inhibit HMGCR, the rate-limiting step of hepatocyte cholesterol biosynthesis. 2. Inhibition of cholesterogenesis leads to enhanced expression of LDL receptors in hepatocytes, resulting in increased uptake of LDL-C (bad cholesterol) from the blood and ultimately reduced plasma cholesterol.

Statin intolerance

Adherence to statin treatment is the cornerstone to ensure better management of and reduced death from CVDs including myocardial infarction (MI) and stroke. However, statin intolerance poses a major challenge to patient adherence to the statin treatment\textsuperscript{10–12}. Several reports have shown that due to statin intolerance, 30% of patients discontinue statins, which limits the effectiveness of stains for patients at risk of CVDs\textsuperscript{13–15}. Based on recent reports, up to 50% of patients taking statins discontinue due to statin intolerance\textsuperscript{16,17}. Consequently, statin intolerance leads to increased risks of reoccurrence of CVDs and associated death\textsuperscript{15,18,19}. Statin intolerance is caused by muscular problems\textsuperscript{14,20}, increased risk for diabetes and obesity\textsuperscript{21}, sleep disturbance\textsuperscript{22,23}, cognitive impairments\textsuperscript{24,25}, and neuropsychiatric effects including anxiety and depression\textsuperscript{26}, although reports are not consistent. Statin-associated muscle problems, also known as statin-induced myopathy (SIM), are the main reason for statin intolerance\textsuperscript{14,27}. Moreover, cumulative reports from the US FDA Adverse Event Reporting System (FAERS) indicate that statin use is associated
with an increased risk of insomnia\textsuperscript{28}. Other studies also reported hallucinations and nightmares with statin use\textsuperscript{29}. In addition to the burden on the health system, a recent study estimated that non-adherence to statins in the US leads to an additional $18.6 billion in annual healthcare costs and 24,000 preventable deaths\textsuperscript{30}. Despite extensive research, the underlying mechanisms for statin-associated myopathy and sleep disturbances remain enigmatic\textsuperscript{14,27}. 
Aims

Given the fact that lipid-lowering agents other than statins do not offer similar optimal therapeutic benefits in the management of CVDs compared to statins, elucidating the underlying mechanisms is crucial to enhance our understanding of molecular events of statin-associated side effects and thus pave the way towards a discovery of statins with fewer side effects or therapeutic interventions to mitigate these side effects. This thesis, therefore, aims to delineate the underlying mechanisms for statin-associated myopathy and sleep problems. Specifically, we investigated the role of the known target for statin, HMGCR, in statin-associated muscle problems and sleep disturbance as well as the underlying mechanisms for such side effects.

Paper I

Paper I aims to decipher the effects of statins on sleep patterns and scrutinize whether these effects result from central inhibition of HMGCR using D. melanogaster.

Paper II

Paper II aims to investigate the association between statin use as well as genetic variants related to HMGCR and PCSK9 with insomnia risk and chronotype in the UK Biobank.

Paper III

Paper III aims to first investigate whether statins can induce myopathy-like phenotypes in D. melanogaster. The second aim is to dissect the effects of statins on muscles and scrutinize the role of Hmgcr and skeletal muscle chloride channels in statin-induced myopathy.
Paper IV

Paper IV aims to delineate the temporal relationship between myofibrillar damage and mitochondrial changes associated with statins but also to investigate whether statins-induced impairment of mitochondrial respiration results from inhibition on Hmgr using *D. melanogaster* as a study model.
Materials and Methods

*Drosophila* work

In *D. melanogaster* (fruit flies) and humans, statin-target, HMGCR (in *D. melanogaster*, *Hmgcr* is the homologue of HMGCR), is highly conserved\(^\text{32-34}\). Moreover, *Drosophila* represents a very good model for studying the molecular events related to muscle issues and sleep, with potential implications for human diseases, given the similarity of many physiological and anatomical features of muscles and sleep in flies to those in humans. Hence, in this thesis, we conducted translational studies using *Drosophila* to scrutinize the underlying mechanisms for SIM and sleep problems. Subsequently, we sought to determine whether these effects recapitulate in humans by analysing cohort data from the UK Biobank.

Functional studies: Gene manipulation

UAS-Gal4 is a powerful genetic tool used to control gene expression in a tissue-specific manner. It consists of an upstream activation sequence (UAS) and the yeast Gal4 transcription factor, which, upon binding to UAS cis-regulator sites, activates the transcription of specific genes. By using a muscle-specific promoter, we were able to lower the expression (knockdown) of the gene of interest in the muscles using the UAS-Gal4 system. To mimic the inhibitory effect of the statin on its target, *Hmgcr*, we employed the UAS-Gal4 system and specific muscle promotors to knock down *Hmgcr*, in the tissue of interest, including neurons, PI and muscles. We also observed that statins lowered the expression of skeletal muscle chloride channel, ClC-a (paper III) and thus we mimicked this effect by knocking down *ClC-a* in the muscles. Flies were raised at 25 °C until adulthood, and upon collection, adults were subsequently raised at 27–29 °C for appropriate durations. In all assays, F1 progeny was utilized. To assess the impact of fluvastatin on mitochondrial morphology, we crossed the *Mef2-GAL4* driver with UAS-mito-GFP to express GFP fused to a mitochondrial matrix targeting signal in skeletal muscles and generate *Mef2-Gal4>*UAS-mitoGFP* fly lines.
Fluvastatin concentration

Fluvastatin was used in previous *Drosophila* studies at concentrations of 0.05, 0.5, and 1 mM without reporting toxic effects. We wanted to check if these levels align with reported toxic doses in mice and the typical human plasma concentration after taking a 40 mg dose.

Considering factors like the fly’s weight, daily intake, and human dosage, we found out that 0.5 mM (0.3 mg/kg/day) and 1 mM (0.74 mg/kg/day) are well within the therapeutic range. On the other side, higher concentrations like 20 mM and 40 mM equate to toxic doses in mice as reported by preclinical studies (https://www.accessdata.fda.gov/drugsatfda_docs/label/2022/201635s029lbl.pdf accessed on 1 Jan 2024). Our calculations revealed that fluvastatin concentrations of 20 mM (14.7 mg/kg/day) and 40 mM (29.5 mg/kg/day) in *Drosophila* are equivalent to reported toxic cancerogenic doses in mice. Considering the regular human doses of 40 mg/day and a maximum of 80 mg/day, and the average human male weight of 70 kg, daily doses per kg are 0.57 mg/kg/day for 40 mg and 1.1 mg/kg/day for 80mg. Consequently, we concluded that concentrations of 0.5 mM (0.3 mg/kg/day) and 1 mM (0.74 mg/kg/day) fall within the therapeutic range, making them suitable for our study (Fig. 2).

![Fluvastatin concentration graph](image)

**Fig. 2** The equivalent dose for the fluvastatin in *Drosophila* in mg/kg body weight/day where the concentration between 0.5 and 1.2mM falls within the therapeutic dose of humans.

In fluvastatin groups, we fed W1118, CSORC or Mef2-Gal4>UAS-mitoGFP flies with equivalent fluvastatin mixed with food while in control groups, we fed flies with normal food for either 24 hours, two and five days.
Quantification of locomotion and climbing activities

*Drosophila* Activity Monitoring System (DAMS) was employed to quantify the general locomotion activities, namely the activity counts per 30 minutes and total activity as described previously."""". We employed the Forced-Climbing System described by Moulin et al."""". We have improved the system to measure hourly locomotor activity (moves), speed per hour, and maximum position reached (position) which was described in detail in our method section of the third paper."""".

Immunohistochemistry and morphology examination

In order to explore the impact of fluvastatin on the ultrastructure of muscles, transmission electron microscopy and confocal microscopy were used to examine the morphology of myofibril and mitochondria of the skeletal muscles of the legs and thorax for both fluvastatin-treated flies and mutant flies. We stain the muscle with immunogold particles of chloride channel using Rabbit Anti-ClC-2 Antibody (Abcam, The Netherlands,#ab192506) at 1:500 dilution. ImageJ was used to measure the individual myofibril diameters and thickness, the space between myofibrils and mitochondrial surface area, and the number of round-shape mitochondria. To stain the mitochondria, we used rabbit anti-Mef2 (1:200, Eileen Furlong, Heidelberg, Allemagne), Alexa-conjugated phalloidin (1:200, Thermo Fisher, Waltham/Massachusetts) and goat anti-GFP (1:200, Abcam, ab6673). The quantification of the mitochondria content was carried out according to Avellaneda et al."""". For the western blot analysis, anti-CLC-a antibodies, Rabbit Anti-CLC-2 Antibody (Abcam, The Netherlands,#ab192506) at 1:500 dilution; β-Actin polyclonal Antibody (ThermoFisher #PA5-16914) at 1:1000 dilution were used. The whole body of the flies was used in the western blotting analysis.

Quantification of gene expression

To investigate the effect of fluvastatin on the key genes involved in myopathy and lipid metabolism to gain insights into the molecular mechanisms, we employed quantitative polymerase chain reactions, qPCR, a powerful molecular biology technique that enables precise measurement of gene expression levels. Through qPCR, we were able to quantify the mRNA levels of specific genes known to be associated with myopathy in fluvastatin treatment. This allowed us to elucidate changes in gene expression induced by fluvastatin, providing valuable insights into the molecular mechanisms underlying statin-associated myopathy. The whole body of ten CSORC or W1118 flies was used in each sample. RNA was extracted and transcribed to DNA using a polymerase chain reaction as described previously."""". The purity and levels of the RNA and
cDNA were determined using nanodrop in a Multiscan machine (Thermo Scientific™) using μDrop™ Plate. Then, cDNA was diluted in a ratio of 1:30 and gene expression was measured using qPCR.

Mitochondrial respiration

To examine the impact of statins on mitochondrial respiratory functions, we employed high-resolution FluoRespirometry which allowed us to measure the reactive oxygen species (ROS) and the efficiency of fatty acid oxidation (FAO). In the high-resolution FluoRespirometry study, thorax muscles were dissected and then permeabilized for mitochondrial respiration and H₂O₂ production measurements using the Oxygraph-2k system. The experimental setup included substrate-uncoupler-inhibitor titration with various compounds such as pyruvate, malate, ADP, proline, succinate, glycerol-3-phosphate, CCCP, rotenone, malonate, and antimycin A.

In the assessment of fatty acid oxidation (FAO)-dependent mitochondrial respiration, palmitoylcarnitine and malate were used as substrates, with subsequent additions of ADP, pyruvate, additional malate, proline, succinate, glycerol-3-phosphate, rotenone, malonate, and antimycin A. The calculation of the flux control factor was performed to determine the contribution of each substrate to the respiration rate, as well as the oxidative phosphorylation (OXPHOS) and electron transfer (ET) coupling efficiencies.
Cross-sectional observation study

Data set
The cohort data resource was obtained from the UK Biobank, with over 500,000 participants between 2006-2010. The baseline data covers a diverse range of variables, including insomnia, chronotype, demographics, lifestyle factors, and various health-related parameters. After excluding individuals with non-white ethnicity, genetic-related issues, neurological/psychiatric disorders, or a history of stroke, we obtained 206,801 participants.

Genetic analysis
In our genetic analysis, we focused on three specific single-nucleotide polymorphisms (SNPs) known to be associated with lower cholesterol regulation: rs17238484 and rs12916 in the HMGCR gene, which serve as proxies for Hmgcr inhibition typically induced by statin medications, and rs11591147 in the PCSK9 gene, acting as a proxy for PCSK9 inhibition typically observed with PCSK9 inhibitors. By examining these genetic variants, we aimed to indirectly assess the potential impact of statins or PCSK9 inhibitors treatment on cholesterol levels and related health outcomes in our study population. To ensure the reliability of our genetic analysis, we evaluated the equilibrium of these genetic variants using the Hardy-Weinberg equilibrium, a fundamental principle in population genetics that helps to validate the accuracy of genotype frequency estimations and enhance the robustness of our findings.

Statin treatment and sleep patterns
Participants' self-reported statin usage showed that the majority of patients were on lipophilic statins (93%), with atorvastatin and simvastatin being particularly prominent. Among the lipophilic statins, simvastatin represents 79.0%, atorvastatin 20.7%, and fluvastatin 0.3%. Sleep patterns were assessed through a touchscreen questionnaire, which asked the participants about their experience of insomnia and chronotype, and evaluated whether participants identified as morning, evening, or in-between persons.
Statistical Analysis

IBM SPSS version 26 was utilized for data analyses. Binary logistic regression analyses were applied specifically for assessing insomnia, while logistic and linear regression were employed for evaluating other associations. To address the issue of multiple comparisons and control the family-wise error rate, the analyses were adjusted accordingly, with a significance threshold set at \( p < 0.025 \). This threshold was chosen to mitigate the risks associated with conducting numerous statistical tests simultaneously, reducing the likelihood of false positive results due to random variability. Furthermore, interaction effects between statin treatment and genetic variants were explored to understand the interplay among statin use, genetic factors, and sleep-related outcomes within the broader context of the health dataset.
Results

Paper I

Pan-neuronal Hmgcr knockdown recapacitates the fluvastatin sleep-promoting effect
To examine the impact of fluvastatin on sleep parameters, we fed wild-type flies, W1118, with fluvastatin at gradually escalating doses for five consecutive days and then assessed sleep patterns using DAMS. Our data showed that in a dose-dependent pattern, fluvastatin induced prolonged sleep duration in three parameters: prolonged sleep episode duration, decreased number of sleep episodes and shortened total sleep onset latency. To determine whether the fluvastatin sleep-promoting effect is mediated by the inhibition of statin target, Hmgcr, we carried out pan-neuronal Hmgcr knockdown using an elav-GAL4 driver. Intriguingly, similar to fluvastatin treatment, pan-neuronal Hmgcr knockdown led to prolonged sleep duration in all three parameters.

Central Hmgcr knockdown in pars intercerebralis (PI) promotes sleep, comparable to fluvastatin treatment and pan-neuronal Hmgcr knockdown
While it is expressed in all neurons, Hmgcr expression in pars intercerebralis is very high, specifically in insulin-producing cells (IPCs) in flies. Since Hmgcr in this area has been shown to regulate feeding behaviour in flies, we asked whether Hmgcr in this area is also responsible for statin-mediated prolonged sleep in flies. To answer this question, we knocked down Hmgcr in the PI IPCs using the Dilp2 driver. Intriguingly, similar to fluvastatin and pan-neuronal Hmgcr knockdown, loss of Hmgcr in Dilp2 neurons caused a prolonged sleep duration, decreased total sleep episodes and reduced sleep latency, which are more strongly at night.

Loss of Hmgcr in the PI DH44 neurons changes sleep pattern opposite to global Hmgcr inhibition (pan-neuronal Hmgcr knockdown and fluvastatin treatment) or Dilp2 Hmgcr knockdown
DH44 is a Drosophila homologue of human Corticotropin-Releasing Factor (CRF) and its expression is restricted to six neurons within the PI of flies. It plays a crucial role in the regulation of the rhythmicity of locomotion and circadian rhythm in general. In order to examine whether the inhibition of Hmgcr in this area contributes to the sleep-promoting effect of fluvastatin, we
knocked down *Hmgcr* in PI DH44 neurons using a *DH44-GAL4* driver. Surprisingly, DH44 *Hmgcr* knockdown resulted in reduced sleep duration and enhanced sleep latency, which is contrary to global Hmgcr inhibition or central Hmgcr inhibition.

**Hmgcr controls sleep consolidation irrespective of the circadian rhythm**

Sleep regulation is primarily governed by the intricate interplay between hemostatic and circadian mechanisms. Since DH55 neurons have been associated with the regulation of circadian rhythm and clock neurons, we asked whether *Hmgcr* plays a role in sleep regulation through these mechanisms. Initially, we subjected *Hmgcr* knockdown flies (induced by elav, Dilp2, and DH44) to constant darkness for five days and assessed their strength and the period of free-running circadian locomotor rhythms. Loss of *Hmgcr* in pan-neurons, PI IPCs, or PI DH44 revealed a typical manner in both the strength and the duration of free-running circadian locomotor rhythms when compared to controls. This suggests that the sleep-modulating effect of *Hmgcr* is independent of the circadian rhythm.

**Paper II**

**Statin use is associated with an increased risk for insomnia differently from genetic variants for HMGCR and PCSK9**

28.3% of the participants (mean age 56 ± 7.99 years) reported insomnia between 2006-2010 where insomnia prevalence was higher in women (32.2%) compared to men (23.8%). Statins were associated with a 7% increased risk for insomnia with $P = 1.42 \times 10^{-4}$. In contrast with statin use, HMGCR rs17238484-G risk allele was associated with a 2% decreased risk for insomnia with $P=0.045$. Similarly, HMGCR rs12916-T was associated with a 2% lower risk for insomnia with $P=0.009$. Consistent with statin use, PCSK9 rs11591147-T was associated with a 7% higher risk for insomnia with $P=0.001$. Regarding chronotype, neither statin treatment nor HMGCR gene variants were associated with chronotype, while PCSK9 rs11591147-T was associated with a 17% increased risk for evening preference with $P=0.007$.

**Paper III**

**Chronic fluvastatin treatment reduces climbing and general locomotion activities**

Statins have been shown to affect the muscles in different study models. Hence, we first asked whether fluvastatin influences locomotion and climbing ability. To answer this question, we treated CSORC flies with fluvastatin for
five days and then employed the Force-Climbing System to measure different parameters of climbing, including moves, speed, and position (a scale of 16 positions in the tube indicates how far flies can climb), as well as DAMS to measure general locomotion activities including activity counts per 30 min. and total activity counts. Both 0.5mM and 1mM fluvastatin-treated groups displayed significantly lowered climbing speeds and moves, compared to control groups, while only the 1mM fluvastatin group exhibited a significant reduction in position. Consistent with our previous study, chronic fluvastatin treatment induced lowered general locomotion activity without changing activity while awake. However, acute fluvastatin treatment for 24 hours did not affect the climbing activities. Taken together, these findings suggest that chronic fluvastatin treatment reduced locomotion and climbing activities in flies. According to these results, we treated flies with 1mM fluvastatin for five days for the rest of the experiments in our studies to ensure consistency.

**Chronic fluvastatin treatment causes myofibril damage and mitochondrial phenotypic changes along with impaired muscle regeneration and development**

Next, we asked whether fluvastatin-induced impaired locomotion and climbing ability are initiated by damage to the muscles. To address this question, after treating flies chronically with fluvastatin for five days, we examined the morphology of the skeletal muscle of the legs under TEM with consideration that myofibril integrity and altered sarcomere diameter as features of myopathy, which is in accordance with previous studies.

Intriguingly, the fluvastatin-treated group exhibited severe myofibril damage evinced by disorganized myofibrils, skewed and long sarcomeres and Z-line streaming, along with vacuoles and a few myelin figures. These myelin figures are similar to previously reported toxic myopathy in humans. In terms of mitochondrial morphology, the fluvastatin-treated group displayed mitochondrial phenotypic changes where rounding-up, enlarged abnormal shape and scattered mitochondria were observed.

To determine whether fluvastatin-induced muscle damage is associated with impairment of muscle growth, we examined the transcripts of the Mhc (Myosin heavy chain), Mef2 (myocyte enhancer factor-2) and Zfh1 (Zn finger homeodomain 1) since there are involved in muscle development, proliferation, and regeneration. Fluvastatin-treated group showed a remarkable reduction in Mhc, Mef2 and Zfh1 expression, in comparison with the control. Collectively, chronic fluvastatin treatment is associated with muscle damage including myofibrillar damage, longer sarcomeres and mitochondrial phenotypic changes, as well as impaired muscle development and regeneration.
Chronic fluvastatin treatment induces lipotoxicity and impairs insulin signalling

Previous reports have shown that the muscle biopsy of statin-treated patients revealed disruption of certain key genes in lipid metabolism and insulin signalling. Therefore, we asked whether chronic fluvastatin treatment impairs key genes of lipid metabolism and insulin hemostasis. Strikingly, the expression of the vast majority of the genes involved in lipid metabolism after chronic 1mM fluvastatin treatment was significantly downregulated, notably Lpin, Dgat2 (Diacylglycerol O-acyltransferase 2) and Hnf4 (Hepatocyte nuclear factor 4-gamma), compared to control. The expression of the key genes involved in insulin hemostasis, Chico (encodes an insulin receptor substrate) and Thor (encodes a eukaryotic translation initiation factor 4E-binding protein) were also remarkably reduced. Altogether, fluvastatin treatment in flies impaired lipid metabolism and insulin homeostasis.

Loss of Hmgcr in the skeletal muscles induces mitochondrial phenotypic changes and lowered locomotion activity similar to fluvastatin

HMGCR is the main target of statins and several studies have linked the SIM with the inhibition of HMGCR and subsequent reduction of isoprenoids and CoQ10, although data are inconclusive. Therefore, to elucidate the role of Hmgcr in muscle damage, we selectively knocked down Hmgcr in the skeletal muscles using Mhc-GAL4 driver. Compared to control groups, Hmgcr knockdown flies displayed reduced general locomotion activities, and enhanced climbing position without changing climbing speed and moves. In terms of muscle morphology, Hmgcr knockdown flies displayed mitochondrial phenotypic changes including scattered and roundup shapes whereas mitochondrial areas were not altered, all without noticeable alteration in the myofibril integrity. However, sarcomere lengths of Hmgcr knockdown flies were higher than one control (Mhc-Gal4>W1118) group and lower than another control group (W1118>UAS HmgcrRNAi).

Compared to fluvastatin treatment, loss of Hmgcr in skeletal muscles resulted in comparable reductions in locomotion and the presence of scattered round-shaped mitochondrial phenotypes. Nevertheless, loss of Hmgcr did not compromise climbing ability or induce myofibrillar damage or elongated sarcomeres. This suggests that inhibiting Hmgcr in skeletal muscles may not be the primary cause of the myofibrillar phenotypic changes associated with chronic fluvastatin treatment.

Specific knockdown of ClC-a in skeletal muscles triggers myofibrillar phenotypic changes and impaired climbing ability similar to fluvastatin

Selective Hmgcr inhibition in the skeletal muscles did not trigger myofibrillar damage or change sarcomere lengths; therefore, we hypothesized that other
mechanisms are involved. Recent reports have revealed that fluvastatin reduces the expression of the skeletal muscle chloride channel CLC-1, which is encoded by the CLCN1 gene, in mammals. As a result, we sought to examine whether inhibiting the CLCN1 homolog in flies could mediate myopathy-like characteristics. Based on literature and the advanced orthologue prediction tool Phylome DB, ClC-a is the most closely related homolog to human CLCN1. Thus, we investigated the impact of fluvastatin on ClC-a expression. First, we employed immunogold double labelling of ClC-a using rabbit anti-CLC-2 antibodies and then counted ClC-a gold particles under TEM. As predicted, the fluvastatin group exhibited a remarkable reduction in ClC-a gold particle expression compared to controls. This finding was corroborated by western blot, which demonstrated a significant reduction in ClC-a expression in the fluvastatin group.

So far, no evidence has demonstrated that CLC-1 inhibition in the skeletal muscles causes muscular damage or myopathy phenotypes. Thus, we sought to investigate the effects of ClC-a inhibition on muscle integrity using an Mhc-GAL4 driver to selectively knock down ClC-a. ClC-a knockdown flies exhibited significantly reduced climbing movements and activity while awake, indicating a locomotion defect. However, their speeds, positions, and overall locomotion activities remained unchanged compared to controls. Intriguingly, the muscle morphology of ClC-a knockdown flies revealed a similar pattern to fluvastatin treatment including damaged myofibrils, distorted and misaligned sarcomeres, and Z-line streaming and elongated sarcomere as well as few myelin figures. At the mitochondrial level, rarely did we observe round-shaped mitochondria in ClC-a knockdown flies, but no significant change in the mitochondrial area compared to both control groups. Taken together, fluvastatin-phenotypic changes in the myofibrils and lowered climbing movement might be mediated by inhibition of ClC-a.

**Paper IV**

Fluvastatin treatment induced mitochondrial morphological changes in a time-dependent manner and these changes preceded myofibril damage

In the third paper, we showed that chronic fluvastatin treatment induced myopathy-like phenotypes characterising by myofibrillar damage and mitochondrial morphological changes. The temporal relation between mitochondrial alterations and myofibril damage remains elusive. To elucidate this, we labelled the muscle components with specific markers using Mef2-Gal4>UAS-mitoGFP line and treated them for two and five days. The dissected thorax muscles under confocal microscopy showed that, in the control group, the myofibrils were organized in parallel, and mitochondria adopted a filamentous
shape along the myofibrils. After two days of fluvastatin treatment, the mitochondria exhibited a fragmented network of varying sizes and rounded shapes, accompanied by a reduced overall mitochondrial content. After five days of treatment, the mitochondrial fragmentation became more pronounced, particularly in areas where the myofibrils were more dispersed, and mitochondrial content dramatically reduced. Concurrently, the myofibrils became severely disorganized and separated characterized by thicker diameter and more spacing between individual actin filaments along in a few areas abnormal myofilaments. These findings collectively suggest that fluvastatin treatment initially elicits mitochondrial morphological alterations that precede myofibril damage.

**Chronic Fluvastatin treatment is associated with impaired mitochondrial respiration**

Mitochondrial morphological changes and content are linked to impairment of mitochondrial respiration\textsuperscript{71,72}. Since chronic fluvastatin treatment displayed alterations in mitochondrial morphology including reduced content was remarkably significant and is associated with severe myofibril damage and studies have linked myofibril damage to excessive production of reactive oxygen species, ROS, and reduced mitochondrial content\textsuperscript{73–75}, we asked whether chronic fluvastatin treatment for five days is also associated with impairment of mitochondrial respiration. To address this question, we examined the mitochondrial respiration parameters of the dissected thorax muscles using High-Resolution FluoRespirometry\textsuperscript{76,77}. Chronic fluvastatin treatment significantly enhanced the respiration rate in which $H_2O_2$ production rate was increased significantly, while the $H_2O_2/O$ ratio remained comparable to that of the control group, suggesting that the observed enhanced respiration rate in the fluvastatin-treated group is perhaps caused by enhanced $H_2O_2$ production rate. Taken together, chronic fluvastatin treatment enhances ROS production.

Another parameter of mitochondrial respiration is fatty acid oxidation (FAO). Thus, we asked whether chronic fluvastatin treatment influences the mitochondrial capacity to oxidase fatty acid. Compared to the control group, the fluvastatin-treated group displayed a dramatic reduction in CPT2-dependent FAO-dependent respiration along with a lowered respiration rate and higher $H_2O_2/O$ ratio, suggesting that part of the consumed oxygen is wasted for ROS production. Collectively, chronic fluvastatin treatment triggered a decrease in CPT2-dependent FAO and enhanced the production of ROS.
Discussions and conclusions

The impact of statins on muscles and sleep is well studied, yet data are not conclusive and above all, the underlying mechanisms remain equivocal\(^{22,78}\). Understanding the molecular events behind these side effects is essential to pave the way to discovering new statins with fewer side effects and thus improve adherence and outcomes of treatment of CVDs. Here, we deciphered the molecular events of myopathy and sleep disturbance associated with statins pharmacologically and genetically.

In terms of sleep, we started with *D. melanogaster* to delve into the molecular mechanism of statin sleep-modulating effects and the role of Hmgcr in the regulation of sleep patterns. We found that statin promotes sleep through inhibition of Hmgcr centrally in the PI IPCs, and thus discovered that Hmgcr is crucial for sleep consolidation in flies. To translate these findings, we performed a cross-sectional observation study using UK cohort data and found statin to be associated with increased risks for insomnia, while genetic proxies for HMGCR inhibition showed opposite effects. This data suggests that statin-associated insomnia may be independent of the inhibition of HMGCR, although we have investigated only two HMGCR SNPs, rs17238484 and rs12916, that are well-known for acting as proxies for HMGCR inhibition. Taken together, it is reasonable to postulate that other generic variants of HMGCR that also mimic the inhibitory effect of the statins on HMGCR, such as rs10066707, rs2006760, rs2303152, and rs5909\(^{79}\) which have not been investigated in this study, may also contribute in sleep modulation.

In terms of muscle damage, this is the first thesis using *D. melanogaster* to dissect the molecular events of statin-induced myopathy. We first displayed that statins induced myopathy-like phenotypes that were reminiscent of human myopathy. Specifically, we systematised fluvastatin-associated muscle damage into both myofibrillar damage and mitochondrial phenotypic changes. Mechanistically, we discovered that fluvastatin induced myofibrillar damage through the inhibition of the muscular chloride channel, ClC-a, independently from the inhibition of muscular Hmgcr.

We also delved into the mechanistic pathway through which fluvastatin induces muscular damage. The skeletal muscle chloride channel is regulated by PKC\(\theta\)
(PKC-θ), which upon activation, phosphorylates and inhibits the channel. Our data showed that fluvastatin treatment is associated with the activation of Pkcdelta (Drosophila homolog of PKC-θ), suggesting that ClC-a inhibition might be mediated through the activation of Pkcdelta. Pkcdelta and PKC-θ belong to a novel PKC which is activated through ROS or DAG and is insensitive to Ca^{2+}. Interestingly, our data revealed that fluvastatin impaired mitochondrial respiration, elevating ROS and impairing FAO. We also found that fluvastatin induced lipo-toxicity by disrupting key genes involved in lipid metabolism. Impairment of FAO and lipid metabolism may lead to elevated free fatty acid and DAG, both of which are strong activators of novel PKCs. Thus, we suggest that the fluvastatin-mediated elevation of ROS, FFA, and DAG may activate Pkcdelta, which ultimately inhibits ClC-a in flies (See Fig. 3).

We also investigated whether fluvastatin elevated ROS and impaired FAO through the inhibition of Hmgr. Strikingly, we found that selective inhibition of Hmgr in the skeletal muscles did not alter ROS or FAO, although it resulted in similar round-shape mitochondrial phenotypes to fluvastatin. This suggests that fluvastatin-induced impairment of mitochondrial respiration could be mediated through a mechanism independent of the inhibition of Hmgr.

Fig 3. Statins induce myopathy through differential mechanisms. First, statins inhibit mitochondrial complexes, which leads to ROS overproduction, and impairs lipid metabolism. This results in the accumulation of toxic lipid intermediate metabolites such as FFA and DAG. Both, ROS and lipid metabolites activate novel PKCs, including Pkcdelta (Drosophila homolog of PKC-θ), and ultimately inhibit the skeletal muscle chloride channel and induce myofibril damage.
All things considered, we demonstrate that statin use is associated with increased risk for insomnia while two proxies for HMGCR inhibition protect against insomnia, indicating that statin-associated insomnia might be independent of HMGCR inhibition. In flies, we found a similar pattern of sleep-promoting effects of statins and Hmgcr inhibition. This might suggest that statins modulate sleep in flies differently from humans. We also discovered that statins induce mitochondrial dysfunction and myofibril damage independent of inhibition of Hmgcr in flies. We also mapped out a mechanistic pathway through which statins induce the inhibition of skeletal muscle chloride channels. We also delineate that statin-associated mitochondrial phenotypes precede myofibril damage, which implies a causative relationship between mitochondrial changes and myofibril damage.
Here we provide a good foundation for the effect of statin use and the role of HMGCR inhibition in sleep regulation. To strengthen our conclusion, we have to extend our analysis to include other HMGCR SNPs such as rs10066707, rs2006760, rs2303152, and rs5909 using UK Biobank cohort data. Moreover, statins have been reported to have other off-targets such as mitochondrial complexIII\textsuperscript{88}, PPARα\textsuperscript{89}, ITGAL\textsuperscript{90} and HDAC2\textsuperscript{90}. This will enhance our understanding of the off-target effects of the statins and proxies for these off-target effects, which are available in UK Biobank. Longitudinal analysis of statin use and insomnia as well as using Cox regression to omit the proportional hazards would also enhance our understanding of the long-term use of the statins and sleep disturbance considering the time-dependent factor.

In terms of statin-associated muscle problems, we demonstrate that *D. melanogaster*, which offers an affordable and easily genetically manipulated tool, is a powerful model system to study myopathy phenotypes and molecular events for SIM for future research. We also scrutinize the underlying mechanisms for both statin-associated mitochondrial phenotypic changes and dysfunction as well as for myofibril damage. Since myofibril and mitochondria are connected by the sarcoplasmic reticulum, SR, and statins have been reported to alter SR calcium ATPases and induce detachment of the stabilizing protein FKBP12 from the calcium release channel in the SR\textsuperscript{91,92}. Hence, it would be interesting to investigate the sequential occurrence between SR, mitochondrial change and myofibril damage in *D. melanogaster* and other mammalian study models such as mice and rodents after both, acute and chronic exposure to statins.

Currently, we are investigating the association between statin use and proxies for its target and off-target and neuropsychiatric outcomes, namely anxiety, depression and irritability using cohort data. We are also examining the impact of statins on neurons during development and after maturation, which could shed light on the influence of statin use during pregnancy, lactation and early infancy where neurons are still under development\textsuperscript{93}.

Future statin development should take into account the off-target effects, namely the inhibition of skeletal muscle chloride channels as we demonstrate
that this inhibition is the root cause of the statin-induced myopathy. Clinical studies are recommended to examine the potential of skeletal muscle chloride channels, ClC-1, as an early biomarker of muscle damage. Moreover, our comprehensive work will ultimately contribute to finding some therapeutic interventions to mitigate the statins-associated side effects or discovery of new statins with lower incidence of such side effects.
Acknowledgement

My deepest gratitude goes to my supervisors, Jessica Mwiny, Robert Fredriksson, and Mathias Rask-Andersen, for their unwavering guidance and support throughout my PhD research. Your expert mentorship, constructive feedback, and trust in my abilities have been instrumental in shaping this work and guiding me towards this big milestone. I am particularly grateful for your insights on embracing changes and being an independent researcher, which helped me refine my research approach and elevate the overall quality of my research skills and thesis.

I extend my sincere appreciation to Michael Williams, the best monitor, for his valuable monitorship, leadership, immense support and contributions throughout this research journey. Your insightful comments, suggestions, and encouragement have enriched the content and direction of my thesis, and I am deeply indebted to your expertise and dedication. Your willingness to share your knowledge and expertise has been invaluable to my growth as an independent researcher. I am also grateful to Helgi Schiöth for providing me with the necessary resources for research and support.

I am particularly grateful to Robert Malmgren, Saco-S chair, for his invaluable support which helped me to overcome challenges and pursue my academic goals with confidence.

Words cannot express my deepest gratitude to my Father Hamid Al-Sabri, the role model and the true leader; to my mother, Namma Al-Shaja, the kindest and keenest heart; to my wife, Buthaina Al-Sabahi, the true love and best supporter; to my children, Hebah, Maryam, Shuhd and Kenan who are all the essence of my motivation and driving force to excellence; to my brothers, Mahboob, Anwer, Maher and Sami, and sisters, Afaf and Thekra for their unconditional love, unlimited support, and invaluable belief in my abilities. Your constant encouragement and sacrifices have been the bedrock of my success, and I am forever indebted to you for your unlimited support. Your love and understanding have been my guiding lights throughout this challenging yet rewarding journey.
My heartfelt appreciation extends to my colleague and fellow researcher, especially to Ahmed Al-Sheli, who was a major help in training me when I was a master's student and for his valuable help with my first publications. The same appreciation is also for Hadi Boukhatmi, Nourhene Ammar, Neha Behare and others who have contributed to the publications and provided me with meaningful discussions and shared valuable insights during the challenging phases of this research. I would like also to express my thanks to my best English teacher, Wasim Zaharh, who took my English to another level and to my uncle, Ali, who first taught me the basics of biology.

Finally, I would like to express my heartfelt thanks to all my friends who have stood by me, offering encouragement, camaraderie, and a sense of belonging throughout my academic endeavours, especially Abdulwahed Alobely, Ahmed Joni, Ahmed Eldek, Rami Ayoun Alsoud, Nour Aldin Kahlous and Kimia Hossieni. Your presence in my life has been a source of joy, support, and inspiration, and I am truly grateful for your incomparable friendship.

This thesis is a culmination of the collective efforts and support of many individuals, and I am sincerely thankful for each contribution. Thank you for believing in me and enabling me to pursue my dreams.
References


80. Camerino, G. M. et al. Protein kinase C theta (PKCθ) modulates the ClC-1 chloride channel activity and skeletal muscle phenotype: a biophysical and gene expression study in mouse models lacking the PKC0. Pflugers Archiv European Journal of Physiology 466, 2215–2228 (2014).


Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 2047

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)