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Betulinic acid counteracts the lipid accumulation in *Caenorhabditis elegans* by modulation of *nhr-49* expression

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ABSTRACT

Obesity is an ingrained health problem with a multifactorial origin and a long history, thereby innovations in the treatment strategies are of great importance. In the search of a remedy for excessive weight gain, we have directed our investigations to phytochemicals as valuable bioactive compounds. Betulinic acid (BA), among the other triterpenoids, is known for its anti-inflammatory and anti-neoplastic properties. In addition, a previous study of ours has demonstrated a potent anti-adipogenic effect of BA in human adipocytes. Therefore, we aimed here to further verify the anti-obesogenic effect of BA *in vivo* in *Caenorhabditis elegans*. Induction of lipid accumulation in the nematodes was modelled with glucose-supplemented media, followed by treatment with BA (10–50 μM) or orlistat (12 μM) as a control anti-obesity medication. Oil red O and Nile red staining were applied to provide quantification of accumulated lipids. Analysis of the relative expression of genes, related to lipid metabolism suggested molecular mechanism of lipid-reducing action of BA in *C. elegans*. Treatment of nematodes with BA significantly decreased the lipid accumulation, downregulated desaturases involved in lipogenesis (*fat-5*, *fat-6* and *fat-7*), modulated key transcription factors (*nhr-49* and *hlh-11*) and microRNAs (*miR-60*, *lin-4*, *let-7* and *miR-786*) associated with the lipid metabolism. Collectively, the current research provides additional insight on the molecular mechanism of the BA's anti-obesogenic effect *in vivo*. Furthermore, it validates the potential of BA as a candidate compound in obesity management by reducing lipid accumulation.

1. Introduction

Obesity occurs as a result of a broad range of factors including genetic inheritance and epigenetic changes [1]. Overweight is among the main causes for non-communicable diseases such as cardiovascular, type 2 diabetes, certain types of cancer, reproductive failure and other age-related diseases. In addition, a recently published study reveals that overweight patients are predisposed to multimorbidity [2]. Furthermore, over the last years during the COVID-19 pandemic, increased mortality among the patients with higher body mass index (BMI) and correlation between excess weight and infectious susceptibility have been reported [3]. Hence, obesity is an evolving global health problem affecting both young and adults and its prevalence as well as prevention and therapy are of great socio-economic concern.

Current therapeutic approaches for reduction of body weight are initiated stepwise with lifestyle modifications being the mildest (*e.g.*, dietary restriction or intermitted fasting accompanied by increased physical activity). When these interventions are not efficient,

Abbreviations: BMI, body mass index; BA, betulinic acid; PPARγ, peroxisome proliferator-activated receptor gamma; C/EBPα, CCAAT/enhancer-binding protein alpha; ACC, acetyl-CoA carboxylase; TFs, transcription factors; miRNAs, microRNAs; Ago, Argounate; UTRs, untranslated regions; ORO, Oil red O; NR, Nile red; RT-qPCR, quantitative real-time polymerase chain reaction; NGM, nematode Growth Medium; CGC, Caenorhabditis Genetic Centre; MTT, 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyl tetrazolium bromide; CTCF, corrected total cell fluorescence; sbp-1, sterol regulatory element binding protein; SREBPs, mammalian sterol regulatory element-binding proteins; fat-2, fat-5, fat-6 and fat-7, fatty acid desaturases; pod-2, polarity and osmotic sensitivity defect; fasn-1, fatty acid synthase.

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pharmacotherapy is added. The group of the anti-obesity drugs includes a short list of approved medicines such as the pancreatic lipase inhibitor - orlistat that reduces fat absorption in the intestines or the glucagon-like peptide 1 agonist - semaglutide [2,3]. Administration of food supplements is another frequently applied alternative as a complementary to the abovementioned strategies for obesity management. Morbid obesity (BMI $\geq$ 40) that could not be influenced by dietary or pharmacological methods is suggested to a bariatric surgery as the last therapeutic option [3].

Although myriad challenges are faced in the search for novel anti-obesity medications, a number of potential candidates are under investigation for their efficacy and safety, namely incretin-based therapies, mitochondrial uncouplers [4], as well as many different types of natural compounds [5]. Phytochemicals as constituents of the plant extracts possess eventually an immense potential in obesity management either as monotherapy or as an adjuvant to the existing approved drugs. As a naturally occurring pentacyclic triterpenoid, betulinic acid (BA) is reported to possess hepatoprotective [6], anti-neoplastic effects [7] and anti-obesogenic effect [8,9]. Our previous investigation demonstrated that BA inhibits adipocyte differentiation in human adipocytes through inhibition of major transcription factors involved in this process, including peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer-binding protein alpha (C/EBPα) along with downregulation of the lipogenic enzyme acetyl-CoA carboxylase (ACC) and phosphatidylinositol 3-kinase/protein kinase B signaling [10]. Several *in vivo* studies also have suggested that BA decreases body weight and improves metabolic parameters in experimental animals [8,9]. However, further validation is required to affirm the reported beneficial effects of BA on the lipid metabolism *in vivo* with a view to clarify the molecular pathways involved. Considering the extensive use of *Caenorhabditis elegans* as a model organism in obesity and age-related diseases [11], our current experiment hypothesized that BA could decrease the lipid accumulation in glucose-fed nematodes. Additionally, we have examined the potential modulation of mRNA expression of key transcription factors (TFs) and enzymes involved in lipid metabolism in *C. elegans* [11], which could be a target of anti-obesity therapy.

The complexity of transcriptional regulation could be revealed by analysis of expression profiles of microRNAs (miRNAs). They are small non-coding RNAs (19–25 nt) that inhibit the post-transcriptional expression of mRNAs in association with Argounate (Ago) proteins, by targeting and complementary base pairing to their 3′ untranslated regions (UTRs). The binding of Ago-miRNA to the 3′UTR of mRNA results in a translational repression or mRNA degradation [12]. Recently, the circulating miRNAs emerged as new biomarkers for development of metabolic diseases such as obesity also as potential therapeutics [13]. Here, we have analyzed miRNAs that have been previously associated with lipid metabolism and intestinal function in *C. elegans*.

In order to provide insights into the molecular mechanism of the fat-reducing effect of BA, we cultivated *C. elegans* on NGM, supplemented with glucose (2%) to stimulate the lipid accumulation, along with BA (10, 25 and 50 μM) treatment or orlistat (12 μM) as a lipid-reducing control. In addition, we examined whether BA would alter the nematode physiology by performing phenotypic analyses for lifespan, locomotion and brood size along with viability evaluation. To support our hypothesis and reveal the molecular mechanism of BA action in nematodes, we employed lipid staining techniques, mRNA expression analysis of essential for lipid metabolism of *C. elegans* genes and additional determination of the expression profile of selected miRNAs.

2. Materials and methods

2.1. Materials

Betulinic acid (molecular weight 456.7 g/M purity $\geq$ 98% HPLC; Cat. N^{o} 0031 S) is supplied from Extrasynthese (Genay, France). Nematode Growth Medium (NGM; Cat. N^{o} MBS652667) was purchased from MyBiosource Inc. (San Diego, CA, USA). The LB broth Lennox (Cat. N^{o} L3022), agar powder (Cat. N^{o} 05039), M9 minimal salts (Cat. N^{o} M6030), fluoroshield histology mounting medium (Cat. N^{o} F6182), Oil red O (ORO, Cat. N^{o} O1391), Nile red (NR, Cat. N^{o} 72485), orlistat (Cat. N^{o} O4139), NaOH and glucose were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Reagents and consumables for RNA isolation, gel electrophoresis and quantitative real-time polymerase chain reaction (RT-qPCR) analyses from Bio-Rad Laboratories Inc. (Hercules, CA, USA) were used.

2.2. Caenorhabditis elegans maintenance and treatment

The wild type N2 Bristol *C. elegans* and *Escherichia coli* OP50 were obtained by the Caenorhabditis Genetic Centre [CGC, University of Minnesota, MN, USA, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440)]. The nematodes were grown at 20 °C according to standard procedures on NGM plates seeded with *E. coli* OP50 as a food source.

For the following experiments, a standard hypochlorite bleaching method of gravid adults was used to obtain an age-synchronized worm population. Nematodes were maintained on NGM plates (*ca.* 3000–4000 worms per group) supplemented or not with 2% glucose. Normal *E. coli* OP50 was used as a food source until reaching L2 stage. Then worms were transferred to new NGM plates with or without glucose, respectively, pre-treated with orlistat 12 μM [14] or vehicle (to final concentration of 0.1% DMSO) and BA (10, 25 and 50 μM) or vehicle (to final concentration of 0.2% Tween 80:0.2% absolute ethanol). The difference in the vehicle solutions for the selected treatment originates from the limited solubility of BA. Solubilization of BA with Tween 80:absolute ethanol, as described early [15], allowed proper treatment of the petri dishes with BA. For each analysis, preliminary comparison between Control (+G) and both vehicles (+G) affirmed that there is no significant difference, respectively for all the performed assays (Suppl. Figs. 1, 3, 5, 6). Considering this, throughout the manuscript BA and orlistat-treated groups were compared to Control (+G). Treatment concentrations for BA were selected following a preliminary viability assay (Suppl. Fig. 2).

Increased lipid accumulation was validated with lipid staining techniques (ORO and NR) as comparison between glucose and non-glucose supplemented groups (Suppl. Fig. 3). Treatment with a hybrid combination was performed to evaluate whether BA (50 μM) potentiates the lipid-reducing effect of orlistat (12 μM).

As a food source, the treated dishes were seeded with heat-inactivated *E. coli* OP50 to decrease the influence of bacterial metabolism. When reaching L4 larval stage worms were collected and washed with M9 buffer, then further subjected to lipid staining and RNA isolation, as described in the following subsections.

2.3. Viability of nematodes

In order to assess the viability of the nematodes upon treatment for 48 h, we applied the 3-(4,5-dimethylthiazol-2-yl)− 2,5-diphenyl tetrazolium bromide (MTT) assay [16].

2.4. Phenotypic assays of C. elegans

2.4.1. Fecundity assay

Slight modifications were included to the procedures described earlier [17,18]. Briefly, for estimation of the impact of BA and glucose on worm reproductive capacity, fecundity assay was performed. At least five nematodes from each experimental group were individually transferred to a plate containing NGM without glucose and fed with *E. coli* OP50. After reaching fertility stage, the number of laid eggs from every individual was counted daily until the end of the reproductive period and the experiments were done in triplicates.

2.4.2. Locomotion assay

Bending rate of the worms was performed according to [17,18] with some minor modifications. Following treatment with BA at different concentrations, nematodes in L4 larval stage were placed in a drop of M9 buffer on an NGM plate without OP50 and then were allowed to adapt for 30 s. The number of bending movements during locomotion over 30 s were counted. The assay was performed in triplicates and at least 15 worms were used as a representative fragment for each group.

2.4.3. Lifespan measurement

Lifespan measurement was accomplished as described [18] with some slight modifications. At least 30 worms per group were moved to NGM plates without glucose and fed with OP50 after reaching L4 stage. Then worms were monitored daily and considered dead if they did not respond to touch by a platinum wire. Individuals that crawled out of the plate were censored and not included in the analyses. The data were subjected to Kaplan-Meier survival analysis followed by log-rank test.

2.5. Oil red O staining

Lipid staining was performed with ORO as previously described [19]. The worms (*ca.* 1000–1500 per plate) were collected and then washed three times with M9 buffer, followed by fixation in 60% solution of isopropanol for 5 min. Subsequently, the samples were centrifuged and the supernatant was discarded. Filtered ORO dye solution was added to each worm pellet and samples were incubated for 6 h at room temperature. Stained worms were washed three times with PBS (supplemented with 0.01% Triton X-100) for removing the dye and further visualized with Oxion Inverso OX.2053-PLPH inverted microscope equipped with DC.10000-Pro CMEX camera from Euromex (Arnhem, The Netherlands). The quantification of the stained lipid droplets was measured as an average pixel intensity using ImageJ software and represented as normalized pixel intensity against Control (+G) group.

2.6. Nile red triglyceride staining

Nile red was used for staining lipid droplets and measuring fat deposition in *C. elegans*. After 24 h of treatment with BA, around 1000–1500 L4 larvae were collected and washed three times with M9 buffer, then centrifuged and the supernatant was removed. Isopropanol (40%) was added to the worm pellet, followed by incubation for 3 min at room temperature. The staining procedure was fulfilled according to [20], NR solution (600 μL per sample) was added after removal of the isopropanol fixative. Then samples were incubated for 2 h in dark. Subsequently, the dye was removed and the worms were washed three times with PBS (supplemented with 0.01% Triton X-100) and mounted on slides for further visualization. The nematodes were imaged on confocal system Stellaris 5 with inverted microscope DMi8 from Leica (Wetzlar, Germany). The quantification of fluorescence density was performed *via* ImageJ software and normalized to Control (+G) group and presented as corrected total cell fluorescence (CTCF).

2.7. Gene expression analysis through RT-qPCR

Total RNA was extracted *via* PureZol (Bio-Rad) from around 3000–4000 nematodes per group, treated as described in Section 2.2. Agarose gel electrophoresis and UV spectroscopy were used to determine the integrity and quantity of the extracted RNA. Reverse transcription for mRNAs was performed using First strand cDNA synthesis kit (Canvax, Cordoba, Spain). Expression of mRNAs was quantified by $_{\Delta\Delta}$CT method on the CFX Maestro software (Bio-Rad). The *iscu-1* and *mdh-1* were used for endogenous control for mRNAs and the results were normalized to glucose-supplemented control. Nucleotide sequences of the primers, used for analysis of relative mRNA expression, are provided in Supplementary Table 1.

2.8. RT-qPCR analysis of miRNAs and prediction of miRNA interactions via TargetScan

Reverse transcription for miRNAs was performed using Revert Aid H Minus First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) with the use of stem-loop primers [21]. Expression of miRNAs was quantified by $_{\Delta\Delta}$CT method on the CFX Maestro software (Bio-Rad) and normalized to the Control (+G). Endogenous *U18* [22] and the exogenous control *ath-miR-159a* were used as reference genes. Primers, used for cDNA synthesis and qPCR, are listed in Supplementary Table 2.

Targets prediction of significantly affected miRNA was assessed *via* TargetScan v6.2, based on seed complementarity 8/7/6mer between the mRNA and the miRNA [23].

2.9. Statistical analysis

Statistical analyses were performed in SigmaPlot v11.0 from Systat Software GmbH (Erkrath, Germany) and the data were represented as mean ± SEM. Variations between the experimental groups were calculated by one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test. The level of statistical significance was set at $^{*}p < 0.05$. Survival curves of the different groups were compared using the log-rank test to determine whether there is statistical significance between them. Nile red images are representative of at least three independent experiments.

3. Results

3.1. Neither betulinic acid, nor orlistat influenced the daily progeny production and the total brood size of C. elegans

Many factors could affect *C. elegans* reproductive cycle, including treatment with natural or synthetic substances [24]. Thus, we performed fecundity assay to evaluate whether the early-stage treatment with orlistat or BA and 2% glucose could potentially affect the nematode reproduction and further its egg-laying capacity. Our examination of individual worms previously maintained on glucose-supplemented media and treated with BA in concentrations of 10, 25 and 50 μM or orlistat (12 μM) from L3 to L4 stage did not show any change in the brood size compared to untreated glucose-supplemented group (Fig. 1 A), so no reproductive defects in the adult nematodes were observed.

3.2. Betulinic acid did not alter the bending rate of L4 worms

Locomotion is an act of *C. elegans* nervous system, which consist of 302 cells and 75 neurons responsible for the motor functions [25]. Thus, we examined the bending rate of nematodes (measured in bends for 30 s). Through the described analysis we evaluated whether BA or orlistat could affect locomotion. Obtained data shows no significant difference between the bending rate in Control (+G) group and worms treated with BA (10, 25 and 50 μM) or orlistat (12 μM) for 24 h (Fig. 1B). Therefore, the experimental treatments had no impact on *C. elegans* bend rate capacity.

3.3. Supplementation with betulinic acid in the early larval stages did not affect the lifespan of the nematodes

Several studies suggested inhibitory effect of glucose feeding on the nematode lifespan [26,27]. Moreover, identical to our experimental settings glucose feeding is reported as harmless to the worms [28]. Thus, we have performed a lifespan assay to investigate whether 2% glucose feeding from L1 to L4 stage along with the treatment with different concentrations of BA or orlistat for 24 h have an impact on *C. elegans* lifespan. Obtained results suggest no significant difference between the survival curves of glucose-supplemented control group and the

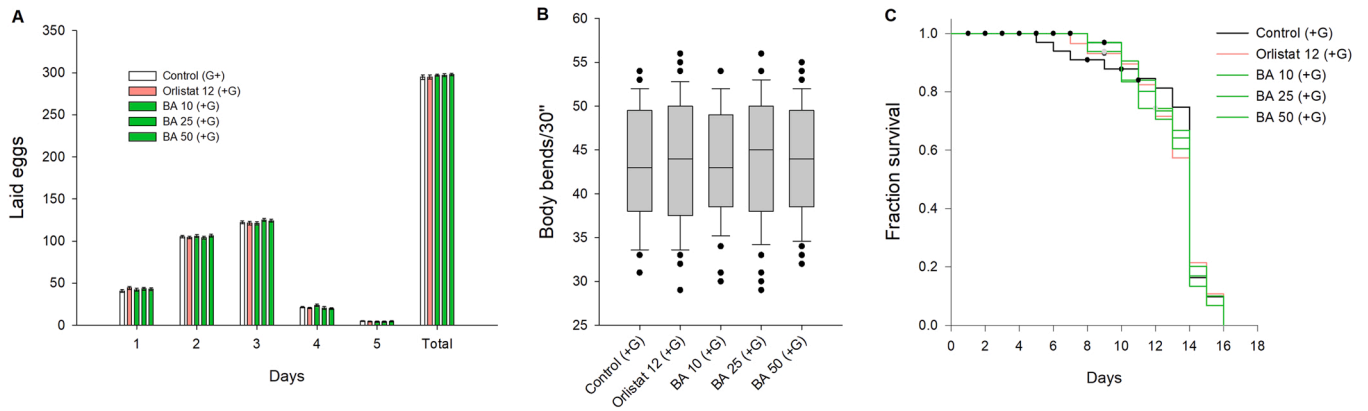

Fig. 1. Betulinic acid (BA) and orlistat did not alter the total brood size, daily progeny production, bending movements and lifespan of *C. elegans*. (A) The effect of BA (10, 25 and 50 μM) or orlistat (12 μM) on the daily and total egg production of *C. elegans* compared to the Control (+G), n = 15. (B) The mobility of BA or orlistat treated glucose-supplemented worms at L4 larval stage, analysed as bend movements within 30 s and compared to Control (+G) group, n = 45. Graph data for (A) and (**B**) are presented as mean ± SEM, *p < 0.05. For comparison between the groups in fecundity and locomotion assays one-way ANOVA, followed by Tukey's *post hoc* test were used. Statistical analysis showed that there was no significant difference between the groups. (C) Lifespan of BA and orlistat-treated worms is compared to glucose-supplemented group and presented as Kaplan-Meier survival curve with log-rank test, n = 30 with no statistical significance between the survival curves.

experimental treatments (Fig. 1 C). Therefore, neither early larval stage glucose feeding nor BA or orlistat treatment in L3-L4 stage altered the lifespan of the nematodes.

3.4. Effect of betulinic acid and orlistat on nematode viability

We evaluated whether orlistat (12 μM) and BA in concentration range from 0.1 to 100 μM could affect nematode viability, through applying an MTT assay. Among the applied treatments only the highest concentration of BA (100 μM) had a minor statistically significant decrease in the nematode viability, compared to the non-treated control group (Suppl. Fig. 2).

3.5. Lipid accumulation in C. elegans was decreased by orlistat and betulinic acid during glucose supplementation

Lipid staining is a rapid assay for estimation of lipid quantification, so both ORO and NR lipid dyes were employed to evaluate the effect of BA on triglyceride accumulation in glucose-fed worms and orlistat as an approved anti-obesity medication. In comparison to humans, *C. elegans* lacks adipocytes, although its fat is deposited as small droplet-like organelles, termed as lipid droplets, found in the nematode intestine and hypodermis [29].

Results from both ORO and NR assays showed similar tendency. Supplementation with 2% glucose to the NGM led to significantly increased lipid accumulation (Suppl. Fig. 3) which is consistent with previous reports on the use of glucose as an obesogenic stimulus in *C. elegans* [30,31].

Nematodes treated with BA 10, 25 and 50 μM exhibited dose-dependent and significant reduction in lipid accumulation assessed by ORO (Fig. 2 C) and NR (Fig. 2B), which did not exceed the effect of orlistat (Fig. 2B, C). The presence of orlistat (12 μM) in glucose-supplemented NGM markedly inhibited lipid accumulation evaluated by ORO (Fig. 2 C) and NR (Fig. 2B). Representative confocal micro-photographs (Fig. 2 A) illustrated the tendency in modulation of fat deposition in nematodes upon the different treatments.

Preliminary NR staining of nematodes treated with both BA (50 μM) and orlistat (12 μM) revealed that the hybrid combination does not potentiate the effect of the substances alone, compared to the glucose-supplemented control (Suppl. Fig. 4).

Our results affirmed the anti-obesogenic potential of BA in *C. elegans*, maintained on glucose-supplemented NGM. Further analyses are applied to investigate the molecular mechanisms involved.

3.6. Betulinic acid altered the expression of genes associated with lipid metabolism in glucose-supplemented nematodes

Lipid metabolism in *C. elegans* involves multiple finely tuned and complexly regulated processes. In the current study we investigated the expression profile of key TFs involved in the activation of the lipid biosynthesis, namely sterol regulatory element binding protein (*sbp-1*) and C/EBP homolog (*cebp-2*). Moreover, as a functional homolog of human PPARs, we also evaluated the TF nuclear hormone receptor 49 (*nhr-49*) which promotes two separate aspects of lipid metabolism - fatty acid desaturation and β-oxidation [32]. In addition, *nhr-49* positively regulates lipogenesis through upregulation of fatty acid desaturases (*fat-2, fat-5, fat-6 and fat-7*) involved in *de novo* lipogenesis [33].

An ortholog of a C/EBPs in *C. elegans* is *cebp-2* [34] that also activates the desaturases. Another important lipogenic TF included in our screening is the ortholog of the mammalian sterol regulatory element-binding proteins (SREBPs) *sbp-1* that participates in lipogenesis through upregulation of *fat-6 and fat-7*, polarity and osmotic sensitivity defect (*pod-2*, homolog of acetyl-CoA carboxylase) and fatty acid synthase (*fasn-1*) [29]. In the current research we also evaluated the helix loop helix-11 (*hlh-11*) TF that supresses the lipid catabolism under well-fed condition. Usually, when the food source is limited, compensatory catabolic processes for energy supply are triggered as upregulation of fasting-responsive adipose triglyceride lipase (*atgl-1*), a lysosomal lipase that breaks down lipid-droplet fats to fatty acids (*lipl-3*), and acyl-CoA synthetase (*acs-2*) related to β-oxidation. Relative expression of the AMP-activated kinase (*aak-2*) as an ortholog of AMP-activated protein kinase (AMPK) was also detected to evaluate whether the treatment affect its metabolic pathway [11].

Betulinic acid considerably reduced accumulation of lipids in glucose-fed nematodes. The lowest BA concentration (10 μM) upregulated *aak-2* (Fig. 3 G) and *acs-2* (Fig. 3H) expression. Interestingly, at transcriptional level BA (10 μM) significantly upregulated *nhr-49*, while the highest concentration applied (50 μM) decreased its relative mRNA expression (Fig. 3 F). Similar biphasic concentration-dependant expression pattern was detected for *atgl-1* (Fig. 3 C). The BA 25 and 50 μM significantly downregulated the fatty acid desaturases (*fat-5*, *fat-6* and *fat-7*) and *pod-2* (Fig. 3 A, I, J, K), while *cebp-2* was upregulated (Fig. 3E). On the other side, *hlh-11* expression levels were significantly increased upon all concentration of BA (Fig. 3D). In addition, lipogenic *sbp-1*, *fat-2* and *fasn-1* were not significantly affected upon all treatments (Fig. 3 L, M, N).

Orlistat treatment triggered significant downregulation of *pod-2*, *atgl-1, hlh-11, nhr-49* and *acs-2* (Fig. 3 A, C, D, F, H), while *lipl-3*, *cebp-2,*

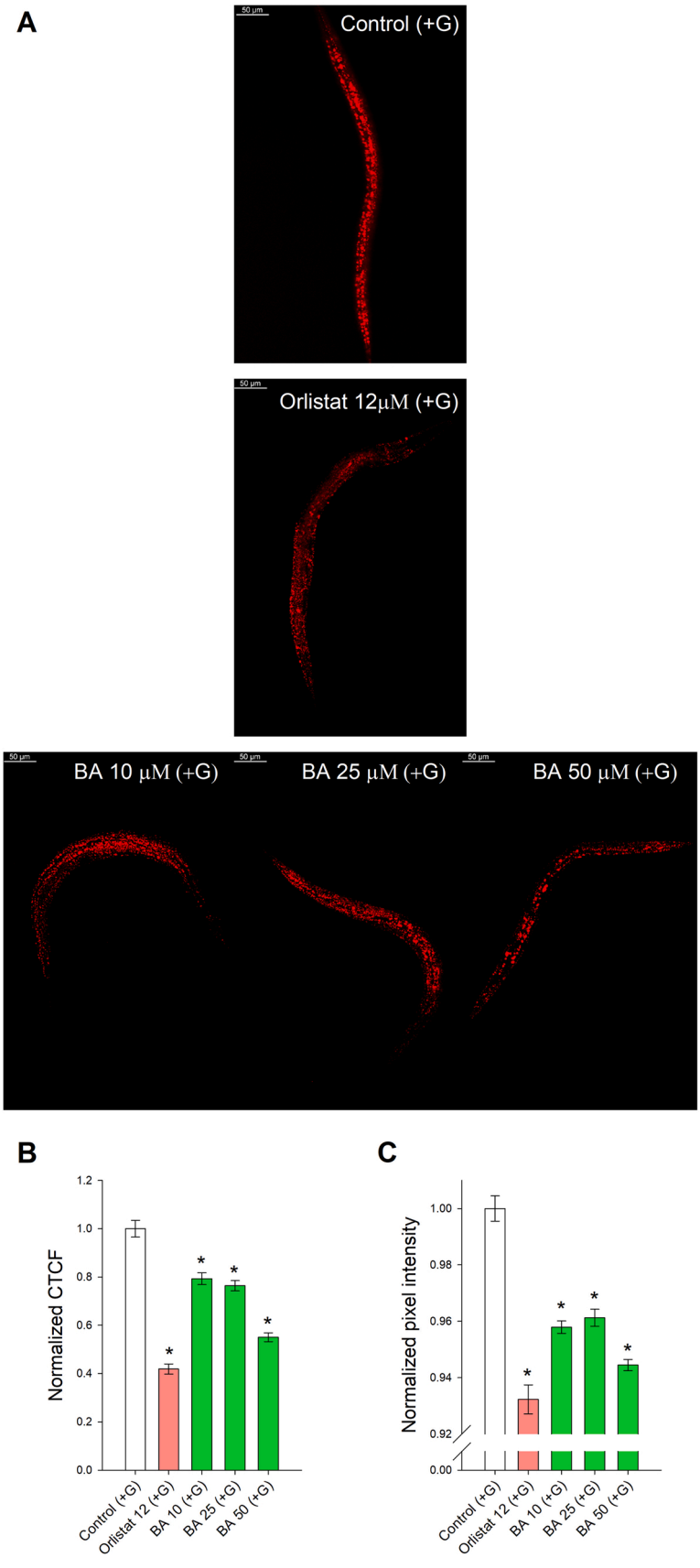

Fig. 2. Betulinic acid (BA) and orlistat reduced the lipid accumulation in glucose-supplemented *C. elegans*. (A) Representative fluorescent images of stained N2 nematodes with Nile red (NR) and treated with BA (10, 25 and 50 μM), orlistat (12 μM) and Control (+G). (B) Quantification of fluorescence intensity of treated nematodes, normalized to glucose-treated control and presented as normalized corrected total cell fluorescence (CTCF), n = 70–80 worms per condition. (C) Quantification of pixel intensity in ORO stained N2 is normalized to the glucose-treated control and presented as normalized pixel intensity, n = 30–40 worms per condition. Error bars indicate mean ± SEM for normalized pixel intensity (for ORO, C) and normalized CTCF (for NR, B) in arbitrary units (a.u.). Statistical significance between the groups were determined by one-way ANOVA, followed by Tukey's *post hoc* test, *$p < 0.05$ compared to the Control (+G) group.

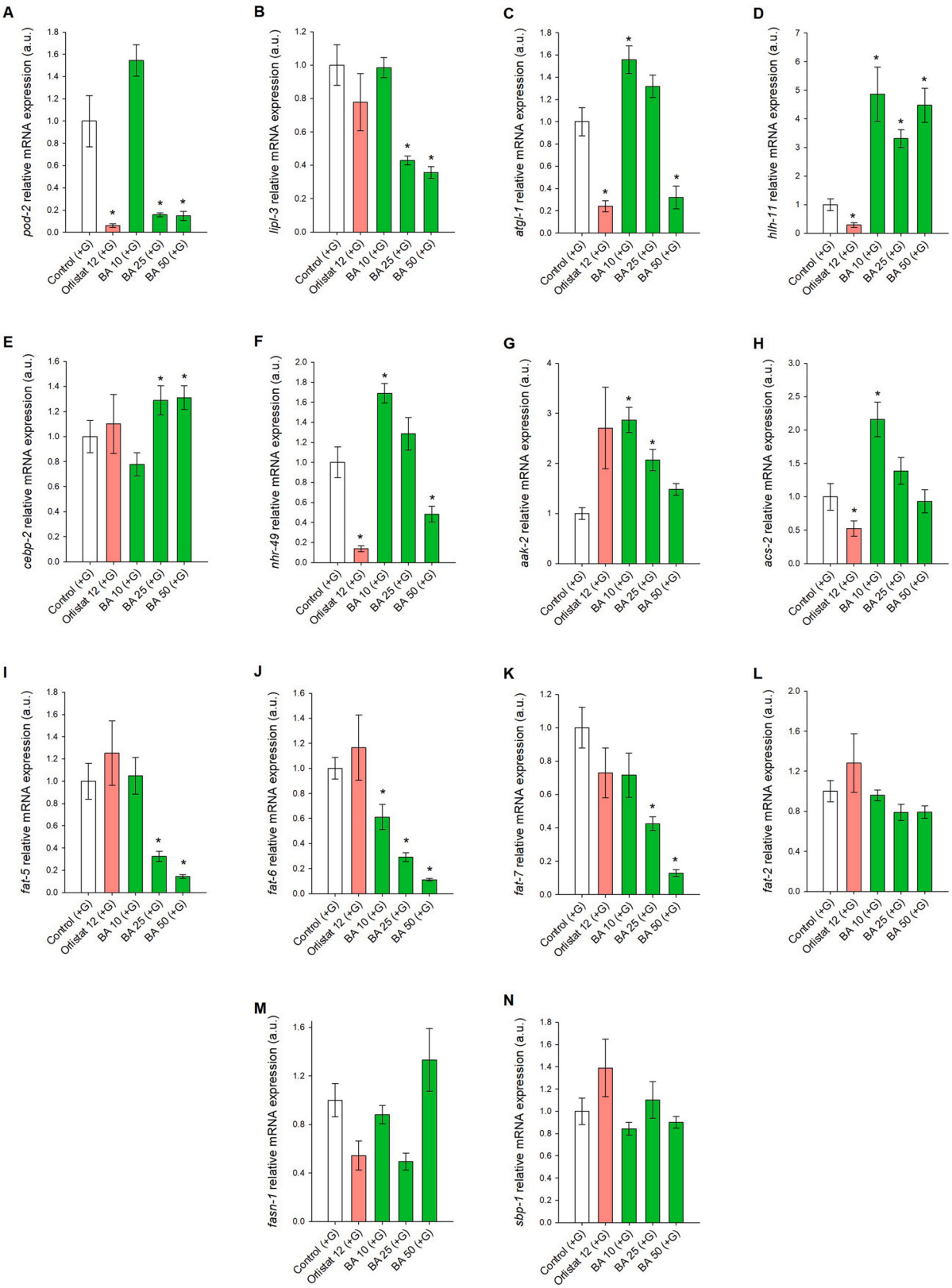

Fig. 3. Expression profiles of genes associated with lipogenesis and lipolysis were modulated upon betulinic acid (BA) and orlistat treatment in glucose-supplemented nematodes. Relative mRNA expression of genes involved in lipid metabolism upon BA (10, 25 and 50 μM) and orlistat (12 μM) treatment, normalized to Control (+G) and represented as arbitrary units (a.u.): (A) *pod-2*, (B) *lipl-3*, (C) *atgl-1*, (D) *hlh-11*, (E) *cebp-2*, (F) *nhr-49*, (G) *aak-2*, (H) *acs-2*, (I) *fat-5*, (J) *fat-6*, (K) *fat-7*, (L) *fat-2*, (M) *fasn-1*, (N) *sbp-1*. The *mdh-1* and *iscu-1* were chosen as reference genes. Error bars indicate mean ± SEM, n = 3 biologically independent samples, with each at least 3 technical replicates, where *$p < 0.05$ compared to the glucose-supplemented control. One-way ANOVA followed by Tukey's *post hoc* test was applied for determination of statistical significance.

aak-2, fat-5, fat-6, fat-7, fat-2, fasn-1 and *sbp-1* didn't show any considerable change in their gene expression (Fig. 3B, E, G, I-N).

The significant transcriptional changes indicated that both BA and orlistat displayed modulatory effect on the nematode fat metabolism. Further analysis of the relative expression of miRNAs was employed in order to a obtain broader picture for the regulation of gene expression upon BA and orlistat treatment in glucose-supplemented *C. elegans*.

3.7. Betulinic acid downregulated the expression of lin-4 and the intestinal miR-60 along with inhibited lipid accumulation

In animals, miRNAs are distinguished by their translational repression of mRNAs. A few studies have lately addressed the potential of natural substances in modulating miRNAs related with cancer, which could be implemented in obesity research as well [35].

Relative miRNA expression profile of (**A**) *miR-60*, (**B**) *lin-4*, (**C**) *let-7*, (**D**) *miR-786*, (**E**) *miR-34* and (**F**) *miR-80* upon betulinic acid (BA - 10, 25 and 50 μM) and orlistat (12 μM) treatment, normalized to Control (+G) in arbitrary units (a.u.). For reference gene *U18* was used and as an exogenous control *ath-miR-159a*. Error bars indicate mean ± SEM, n = 3 biologically independent samples, analyzed in least 3 technical replicates. *$p < 0.05$ compared to the Control (+G), determined using one-way ANOVA followed by Tukey's *post hoc* test.

The RT-qPCR revealed that treatment with BA 10, 25 and 50 μM downregulated the expression of *miR-60* in a dose-dependent manner (Fig. 4A). In our study *lin-4* was also significantly downregulated (Fig. 4B). Interestingly, *miR-786* was found to be significantly downregulated at the lowest concentration (Fig. 4D), while *let-7* shows exactly the opposite manner - considerable downregulation at the highest concentration of BA (Fig. 4C). We also investigated the expression profiles of *miR-34* and *miR-80* but no significant difference in BA-treated groups was detected (Fig. 4E, F). Upon orlistat treatment only *miR-34* was significantly upregulated (Fig. 4E), while *miR-60, lin-4*, *let-7*, *miR-786*, and *miR-80* expression changes did not reach statistical significance (Fig. 4A, B, C, D, F).

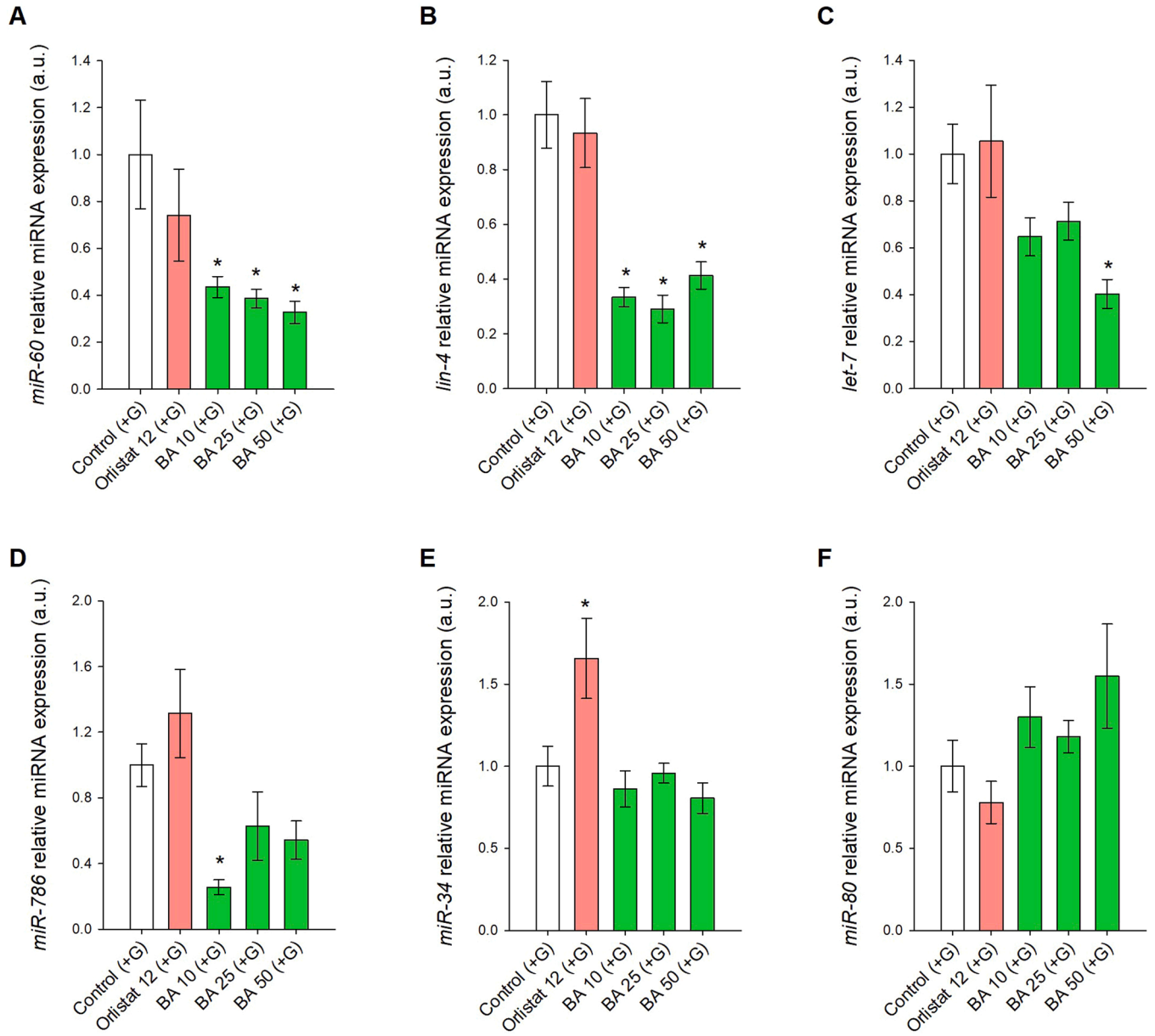

Fig. 4. Expression profile of microRNAs associated with the lipid metabolism.

3.8. In silico target prediction of miR-60 and lin-4

Prediction of miRNA targets is essential in order to understand their biological function. Since we observed significant difference in the expression profiles of *miR-60* and *lin-4* upon treatment with BA we predicted their mRNA targets by employing the TargetScanWorm database [23]. Out of the 403 conserved predicted targets (8mer+1) of *miR-60* we evaluated that three of them are associated with lipid metabolism and could be involved in the mechanism of action – aquaporin-7 (*aqp-7*), *nhr-34* and *acs-4*. Prediction of gene targets for *lin-4* revealed two targets that have been previously reported to be involved in lipolysis *hlh-30* and mediator-15 (*mdt-15*) [36].

3.9. Proposed mechanism of anti-obesogenic effect of BA in glucose-fed C. elegans

The present study aims to elucidate the anti-obesogenic effect of BA that was previously reported [8,9]. Based on our prior research [10] BA suppress adipocyte differentiation and lipid accumulation in human adipocytes. The major lipid regulatory TFs and enzymes included in the study are *nhr-49*, *hlh-11*, *lipl-3*, *acs-2*, and *atgl-1*. The *nhr-49* is involved in fatty acid desaturation and β-oxidation by positively influencing *acs-2*. Overexpression of *acs-2* in the intestine has been reported to decrease the fat accumulation in nematodes [37]. On the other hand, *nhr-49* activates *fat-5*, *fat-6*, and *fat-7*, which downregulation has been associated with decreased fat levels [30].

Betulinic acid decreased lipid accumulation in all concentrations applied according to the lipid staining, however analysis of the relative mRNA expression of *nhr-49* and *atgl-1* suggested different mechanisms involved in BA 10 and 50 μM effect. The lowest concentration BA (10 μM) upregulated *nhr-49*, *atgl-1*, *acs-2* and *aak-2*, thus we could suggest the concentration applied increases triglyceride hydrolysis and β-oxidation. In the view of the fact that among the investigated lipogenic enzymes, only the expression of *fat-6* was slightly affected upon 10 μM BA, we could presume that the lipid synthesis was not affected. On the other hand, the highest concentration of BA (50 μM) revealed predominantly inhibited lipogenesis *via* downregulation of *pod-2*, *nhr-49*, *fat-5*, *fat-6* and *fat-7* along with decreased lipolysis, as suppressed *lipl-3* and *atgl-1* expression.

Assuming that inhibition of lipolysis at 50 μM did not contribute to observed fat reduction, likewise lipogenesis was not affected upon 10 μM, we highlighted the suggested mechanism attributed to observed anti-adipogenic effect of BA in *C. elegans* for both 10 and 50 μM (Fig. 5).

Results from the gene expression along with the observed decrease in the triglyceride content provided a valuable rationale for the outstanding potential of BA as anti-obesogenic molecule. Future clarification of the opposite transcriptional response of *nhr-49* and *atgl-1* triggered by the highest and the lowest concentration of BA is required for detailed explanation of the molecular mechanism of BA.

4. Discussion

Therapy and prevention of obesity and its comorbidities are of great importance [2]. Pandemic-scaled prevalence of this disease demands the exploration of a novel anti-obesity treatment [4]. An interesting alternative to the traditional pharmacotherapy is the application of medicinal herbs and their secondary metabolites in the treatment of metabolic ailments. Besides their potential for obesity management is a scope of numerous scientific investigations [38], great efforts are necessary for development of a novel anti-obesity medication based on the phytochemicals. Among the strategies for obesity management are modulation of lipid metabolism, more specifically decrease of lipid synthesis (lipogenesis) or/and increase in lipid degradation (lipolysis and fatty acid oxidation). Despite both processes are subjected to a complex regulation, they could be assumed as two opposite processes which balance determines the fat accumulation [14].

Caenorhabditis elegans is an adequate model organism for studying human metabolic disorders, such as obesity [32,39]. Considering the resemblance of the *C. elegans* lipid and carbohydrate metabolism to those in mammals, as well as its completely sequenced genome, this tiny nematode is the perfect *in vivo* platform for screening molecules with potential fat-lowering effect [40]. Another great advantage of this model organism is the functional similarity between nematode intestine and human liver and adipose tissue [29]. Numerous compounds of natural origin such as swertiamarin [41], a momordica saponin [30], butein [31], curcumin [42], and polysaccharides from bitter melon [40] have been reported to reduce the fat accumulation in nematodes through affecting essential regulators of lipid metabolism including the transcriptional factors associated with fat synthesis such as *nhr-49* and *sbp-1*. A study also suggests that the natural compounds modulate the expression profile of miRNAs and its monitoring could support the progress in scientific investigation for novel safe and effective therapies [35]. Taking into consideration that targeting miRNAs by phytochemicals in obesity is limited, further investigations are necessary.

Importantly, up to our knowledge, there is no investigation on the effect of betulinic acid on lipid metabolism in *C. elegans*. Supplementation of NGM medium with 2% glucose resulted in increased fat deposition in worms [30,31]. Additionally, we applied orlistat as the most

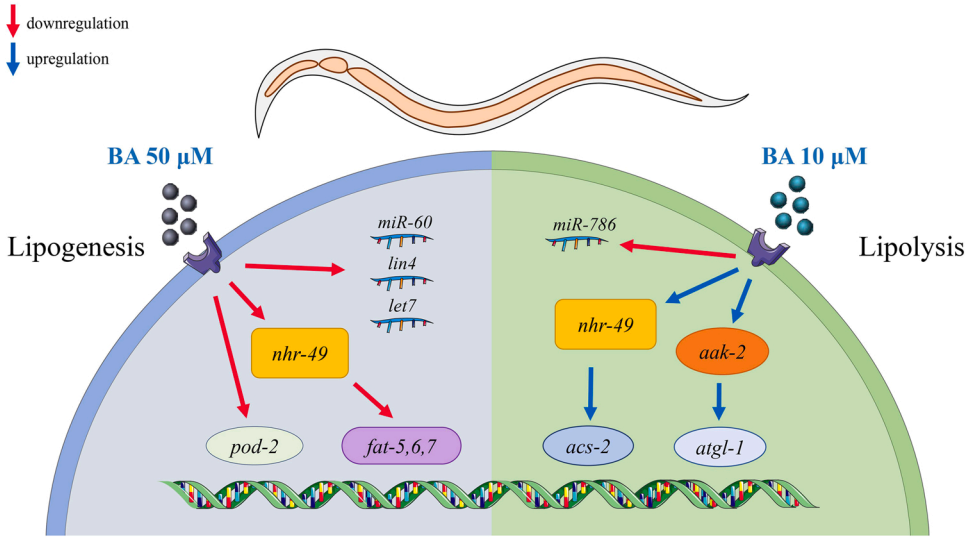

Fig. 5. Reduction in the lipid accumulation in *C. elegans* upon BA treatment is attributed to modulation in *nhr-49* expression profile. Applied concentrations of BA along with glucose supplementation decreased the triglyceride content in the nematodes. Further analysis revealed the difference in the affected molecular pathways between 10 and 50 μM BA. The increased expression of *nhr-49*, *atgl-1*, *acs-2* and *aak-2* in the group treated with 10 μM BA presumes predominantly stimulation of lipolysis. In contrast, the downregulation of *pod-2*, *nhr-49* and its related desaturases *fat-5*, *fat-6* and *fat-7* upon 50 μM BA could lead to inhibited lipid synthesis responsible for the decrease in the lipid accumulation.

commonly used control drug for evaluation of an anti-obesity potential [14,42–45]. Therefore, along with studying the lipid-reducing potential and the mechanism of BA, we provide information about the effect of orlistat on lipogenesis and lipolysis in *C. elegans*. Detailed RT-qPCR analysis suggested mRNAs and miRNAs involved in the anti-obesogenic effect of both BA and the control substance. We further evaluated whether a combinatory treatment with orlistat (12 μM) and BA (50 μM) led to excess in the fat-lowering effect.

As a fat-reducing control in *C. elegans* [14,43,46], orlistat is applied only during the analysis of triglyceride content. The data from the literature is consistent with the currently observed decrease in the lipid accumulation in *C. elegans*. Similar tendency is reported in high-fat diet (HFD) mice models as decreased body weight, BMI and improved serum lipid parameters upon orlistat administration [42–45]. In addition, the current study revealed no significant change upon orlistat treatment in the performed phenotypic analyses.

Analysis of the gene expression in glucose-fed *C. elegans* revealed that orlistat treatment led to downregulation of lipogenesis through modulation *nhr-49*, *pod-2* and *acs-2*. Detected effects partially corresponded to another *in vivo* experimental analysis, where the expression profile of samples from murine epididymal adipose tissue showed significant downregulation of lipogenic enzymes - *ACC, FASN*, and *PPARG* as essential transcription factor for adipocyte differentiation [42]. Orlistat treatment in L3-L4 larval stage in *C. elegans* did not upregulate enzymes related to increased energy expenditure as *acs-2* and *nhr-49*. Inconsistently with our results, such upregulation of related to mitochondrial biogenesis peroxisome proliferator-activated receptor-γ coactivator-1α (*PGC1A*) and *PPARA* is reported for orlistat-supplemented mice subjected to HFD [42]. Assuming that orlistat is pancreatic and gastric lipase inhibitor, among the tested lipases in our study, only triglyceride lipase (*atgl-1*) was downregulated. Regarding the modulation of miRNA expression upon orlistat treatment, notable upregulation in *miR-34* was observed. Considering that *miR-34a* deficient mice are likely to gain weight under HFD [47], whether increase of *miR34* in *C. elegans* decrease susceptibility to overweight is a question that worth extended investigation.

Other pharmacological effects of orlistat, beyond the well-known inhibition of pancreatic lipase, are of a recent scientific interest. Reported data showed that low dose orlistat prevented the major damages caused by ischaemia/reperfusion on brain energy supply, oxidative stress and inflammatory response. Moreover, the leading neuroprotection effect was observed upon the hybrid combination between orlistat and grape seed extract [48]. Also, orlistat inhibits *FASN* and is investigated as an anti-neoplastic agent as an adjuvant to the conventional therapy of adenocarcinomas [49] as well as for its monotherapy [50]. Despite the synergistic combination with orlistat in the context of different biological activities described in the literature, in our experiment such cumulative effect was not observed upon combined treatment with orlistat (12 μM) and BA (50 μM) in *C. elegans*.

Betulinic acid is a plant secondary metabolite with valuable immunoregulatory, anti-inflammatory [51] and hepatoprotective [6,52] properties along with decrease in BMI in *in vivo* models and downregulation of lipogenic enzymes and TFs which could be a basis for application of BA in obesity management [8,9]. According to the literature, betulinic acid has been tested for anthelmintic activity using *C. elegans* as a model system [53]. The concentrations applied for evaluation of anthelmintic activity were far beyond the selected for our experiment. Interestingly, betulin as a precursor in biosynthetic pathway of betulinic acid is reported to possess neuroprotective effect and beneficial outcome on the nematode lifespan [54]. Moreover, performed phenotypic analyses in our experiment revealed that neither glucose in NGM, nor the treatment with BA for 24 h significantly affected lifespan, body bends as well as fecundity of the nematodes. Thus, we could suggest that BA has no ameliorative effect on *C. elegans* lifespan, locomotion and egg-laying capacity despite the effect on the lifespan, reported for betulin as structurally-related to BA substance.

In accordance to significantly decreased lipid accumulation in glucose-fed *C. elegans* assessed by lipid staining in the current study, several investigations, including our former research [10], confirmed the anti-adipogenic effect upon BA treatment in *in vitro* obesity models as reported reduced intracellular lipid accumulation and adipocyte lipid droplets [55,56]. Consistently with the described results from *in vitro* experiments, administration of BA in an HFD-murine model resulted in protection from weight gain, reduced amount of abdominal fat, normalized serum glucose, triglycerides and total cholesterol and restored impaired insulin, leptin and ghrelin levels [8,9].

Regarding highly conserved lipid and carbohydrate metabolic pathways between *C. elegans* and mammals, within the scope of our search we have selected several transcription factors among which - *nhr-49* is a functional homolog to a group of the PPARs [32], proved to be universal modulators of fat metabolism, β-oxidation, fatty acid transport and degradation. Along with results from the previous *in vitro* determination of anti-adipogenic potential of BA [10], inhibition of *PPARγ* was confirmed *in vitro* [55,56] as well *in vivo* [9].

Upregulation of *nhr-49* activates mitochondrial *acs-2* being a key enzyme involved in β-oxidation. Prior investigation affirmed its role in fat accumulation, longevity and hypoxia regulation [57]. Additionally, decreased lipids are reported to be a consequence of increased activity of *acs-2* [37], which was also observed in BA-treated nematodes. Obtained results show significant upregulation of *nhr-49* together with *acs-2* only in the lowest concentration of BA (10 μM), allowing us to suggest a possible fat-reducing effect *via* promotion of β-oxidation. In contrary, the highest concentration of BA (50 μM) revealed significant downregulation of *nhr-49*, thus inhibited lipogenesis. Considering these results and the previously described hermetic nature of phytochemicals [58], we could hypothesise that at transcriptional level treatment with BA induce a biphasic dose-response in regard to *nhr-49* expression.

Triglyceride hydrolysis in *C. elegans* is a complex process regulated by different stimuli and TFs, which is directly related to β-oxidation as a source of free fatty acids (FFAs). The *hlh-11* is a core TF for worm fat degradation, thus was included in the present study along with *atgl-1*, *lipl-3*, and *acs-2* as enzymes whose expression is decreased by *hlh-11* in response to food availability [11]. As expected, loss of *hlh-11* increased *atgl-1* expression [59], together with *lipl-3*, and *acs-2* [11] accompanied by elevated oxidation of FFAs. Fasting-induced lipolysis is regulated by *atgl-1* and *lipl-3* [11], additionally, *lipl-3* is involved in the process of breaking down lipid droplets through lipophagy [36]. Our research indicates stimulated lipolysis measured as elevated mRNA levels of *atgl-1* in the lowest concentration of BA (10 μM) and interestingly, the opposite effect - significant downregulation in highest concentration of BA (50 μM). Surprisingly, *lipl-3* was not affected upon BA (10 μM), while increasing of BA concentration markedly decreased its relative mRNA expression. Comparable tendency between the expression profiles of *atgl-1* and *acs-2* or *lipl-3* is possible due to their common transcriptional regulator, namely *hlh-11*. Observed upregulation of *hlh-11* is partially consistent with observed downregulation in *atgl-1* and *lipl-3* in the highest concentration of BA treatment. Considering the upregulation of *atgl-1*, *lipl-3*, *acs-2*, and *nhr-49* we could speculate that treatment with BA (10 μM) could mimic starvation to nematodes.

In relation to the energy expenditure and partially in accordance with currently presented results in *C. elegans*, BA treatment in murine adipocytes increased the relative mRNA expression of *PGC-1α*, *UCP-2* and fatty acid binding protein. Along with phosphorylation of *AMPKα* its target *ACC* were increased as well, compared to the control group [9]. Moreover, improved muscle energy metabolism upon BA treatment *in vivo* was also revealed as increased expression levels of fatty acid oxidative genes [9]. These findings for adipose and muscle tissues are partially in accordance with results for increased β-oxidation in the lowest concentration of BA applied. The worm ortholog of *AMPKα* subunit - *aak-2* is involved in fat oxidation under starvation, glycogen accumulation, therefore in energy homeostasis [60]. Detected upregulation of *aak-2* in BA (10 and 25 μM) affirms the possible involvement of

β-oxidation in the fat-decreasing mechanism observed in this experimental groups.

Regarding the correlation between *nhr-49* and lipogenesis in *C. elegans*, inhibition of *nhr-49* is followed by a decrease in the expression of *fat-5, fat-6* and *fat-7* genes, which reveals its participation in monounsaturated fatty acids (MUFAs) synthesis [61]. In comparison with declined lipogenic metabolic pathways detected in murine and human models [9,10,55,56], the current findings suggest possible influence of BA (25 and 50 μM) on the biosynthesis of MUFAs assessed by downregulation of the three essential Δ-9 stearoyl-CoA desaturases - *fat-5, fat-6* and *fat-7*. Prior studies emphasized the importance of Δ-9 desaturases not only in the worm lipid metabolism but also in lifespan and fertility [62]. Furthermore, increased expression of these enzymes is suggested to increase the lifespan corresponding with progeny inhibition [33]. Analogous relationship was not revealed within the performed analyses, since downregulation of these desaturases did not shorten the nematode lifespan. In relation to unsaturated fatty acids, *C. elegans* have the unique ability to synthesize poly-unsaturated fatty-acids (PUFAs) *de novo*, with *fat-2* as a catalysator of oleic to linoleic acid conversion [33]. In our study, BA has no effect on PUFAs synthesis considering no significant change in *fat-2* expression upon the treatment.

Our previous study reported decrease of gene and protein expression of *C/EBPα* and adiponectin downregulation of *ACC* upon BA treatment in *in vitro* [10], which did not correlate with obtained data from BA-treated nematodes for *cebp-2*. Possible differences between some of the analyzed transcription factors are attributed to different model system (*e.g.*, human, mice and nematodes).

Key transcriptional factor for the synthesis of fatty acids is *sbp-1* ortholog of human SREBPs is also defined as one of the main regulators of Δ-9 desaturases activity and the genes corresponding to lipogenesis such as *pod-2* (ortholog of a human acetyl-CoA carboxylase), *fasn-1* and *fat-2*. Our analysis on mRNA expression, showed no evidence of *sbp-1* modulation, thus no change in gene expression and association with the mentioned target genes. Additionally, *pod-2* is significantly downregulated in two of BA-treated groups (25 and 50 μM). Thus, we could suggest that *de novo* synthesis of fatty acids is inhibited by BA.

Moreover, we observed downregulation of *miR-60*, which corresponds with previous work and proves that loss of the miRNA significantly reduces body fat content [63] and prolongs the lifespan during mild and long-term oxidative stress conditions caused by paraquat. Further, six miR-60 targets involved in the endocytosis machinery have been described and validated using RNAi [64]. In the context of our study, we focused on predicting and filtering targets associated with lipid metabolism with the use of TargetScan 6.2. Three predicted conserved targets of *miR-60* are thought to be involved in the lipid modification. Aquaporin channel-7 (*aqp-7*), which is involved in transport of water and glycerol is found to be regulated by PPARγ in human and murine adipocytes [65], thus indicating their importance in lipolysis and lipogenesis. The *nhr-34*, less studied TF expressed in the intestine and thus predicted to participate in the lipid metabolism. Acyl-CoA synthetase-4 (*acs-4*) localized on the surface of lipid droplets in the intestine and hypodermis [66]. Downregulation upon BA treatment within all concentrations applied was also observed in *lin-4*. Predicted targets of *lin-4* are *hlh-30* and *mdt-15*, both involved in lipolysis in *C. elegans* [36]. We could hypothesize that BA lowers fat accumulation by downregulating the expression profile of *miR-60* and *lin-4*. In addition, the applied *in silico* prediction provided additional possible targets that could attribute to the observed decrease in triglyceride content in nematodes.

In conclusion, our observations suggest that BA (50 μM) decreased lipid accumulation mainly *via* affecting lipogenesis through downregulation of *pod-2*, *nhr-49* and subsequent Δ-9 downregulation stearoyl-CoA desaturases, while in the lowest concentration applied (10 μM) BA decreased lipid accumulation rather by increased lipolysis and β-oxidation revealed by upregulation of *atgl-1*, *nhr-49*, *aak-2* and *acs-2*. Although the differences in the concentration response on transcriptional level, the outcome in regard to lipid staining had the same attenuation tendency. Collectively, obtained results support our prior hypothesis that betulinic acid is a promising candidate in obesity pharmacotherapy.

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Martina S. Savova: Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Monika N. Todorova:** Conceptualization, Methodology, Data curation, Writing – original draft, Visualization, Investigation. **Apostol G. Apostolov:** Conceptualization, Methodology, Data curation, Writing – original draft, Visualization, Investigation. **Galina T. Yahubyan:** Conceptualization, Methodology, Writing – review & editing. **Milen I. Georgiev**: Conceptualization, Methodology, Supervision, Funding acquisition, Writing – review & editing.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.113862.

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