

# The effect of metformin on autophagy by LC3 expression in Type 2 Diabetes Mellitus (T2DM) human skeletal muscle cell culture



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## ABSTRACT

**Background:** Skeletal muscle is an essential tissue in glucose metabolism. Reduced autophagic capacity to remove damaged contractile proteins in skeletal muscle cells will contribute to the loss of muscle mass. Metformin is the first-line agent for treating type 2 diabetes mellitus (T2DM) patients. This study aims to investigate the effects of metformin on autophagy through LC3 expression in human skeletal muscle cell culture (SkMC).

**Methods:** The T2DM human SkMC was obtained from T2DM treatment naïve patients, purchased from AcceGen Biotech®. Fully differentiated myotubes were randomized into the control and treatment groups. The treatment group was given Metformin in three doses (1 mM, 2 mM, and 3 mM). An immunoblotting assay of AMPK $\alpha$  and LC3 was performed using electrochemiluminescence (ECL). The quantitative expression of AMPK $\alpha$  and LC3 were measured at baseline, after 24-hour, 48-hour, and 72-hour. Data were analyzed using SPSS version 22 for Windows.

**Results:** AMPK $\alpha$  and LC3 expression were higher in the treatment group compared to the control group. The levels of AMPK $\alpha$  and LC3 expression in the treatment group increased dose-dependent. Linear regression analysis demonstrated a significant correlation between metformin administration and LC3 expression levels ( $p < 0.0001$ ).

**Conclusion:** Metformin administration on T2DM human SkMC resulted in increased autophagic activity, marked by increased LC3 expression.

**Keywords:** type 2 diabetes mellitus, skeletal muscle, autophagy, Metformin.

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## INTRODUCTION

Skeletal muscle plays an important role in glucose metabolism.<sup>1</sup> During muscle contraction, the blood glucose levels in the peripheral blood vessels decrease. This is evidenced by a previous study that indicated an association between muscle strength and blood glucose control.<sup>2</sup> Insulin triggers glucose uptake by muscle cells through the Glucose transporter (GLUT) and Sodium dependent glucose transporter-2 (SGLT-2).<sup>3</sup> A greater muscle mass and GLUT4 expression were found in the trained muscles and associated with greater glucose uptake into the muscle. Meanwhile, a decrease in muscle mass and GLUT4 expression in unhealthy muscles diminish glucose uptake.<sup>4</sup> Older patients with T2DM have a two-fold muscle mass loss compared to the elderly without T2DM.<sup>5</sup> Due to this important role in

glucose utilization, loss of skeletal muscle mass (sarcopenia) will worsen insulin resistance (IR) in T2DM.<sup>6</sup>

Macroautophagy (autophagy) is a process in a cell characterized by developing a closed double-membrane vesicle called the autophagosome. The autophagosome then fused with the lysosome to degrade its content. Autophagy largely engulfs a portion of the cytoplasm non-selectively (bulk). However, it can also selectively target dysfunctional organelles or harmful proteins through specific adaptor proteins.<sup>7</sup> This selectivity may preserve cellular function in many tissues during aging. Previously, autophagy was assumed to increase protein degradation; therefore, it might result in sarcopenia. Recent studies have indicated that autophagy maintains muscle mass. Reduced autophagic capacity to remove damaged contractile proteins and dysfunctional organelles contribute to

age-related decline in myofibril function and muscle strength in humans.<sup>8</sup>

Type 2 Diabetes Mellitus (T2DM) is a metabolic disorder that developed due to pancreatic islet  $\beta$ -cells' failure to sustain the hyperinsulinemia required to compensate for IR.<sup>9,10</sup> Insulin resistance diminishes autophagy signaling in skeletal muscle cells. Decreased autophagy will affect the function of insulin-sensitive tissues, including skeletal muscle, and autophagy-deficient skeletal muscle displays similar characteristics as insulin-resistant muscle, such as mitochondrial dysfunction. The mechanism by which IR affects autophagy might be due to an intrinsic defect in autophagy or a response to hyperinsulinemia.<sup>11</sup> Previous study has demonstrated reduced autophagy response to metabolic stress in human muscle precursor cells from humans with T2DM.<sup>12</sup> Insulin has been linked to

inhibition of autophagy through activation of mammalian target of rapamycin (mTOR) which is a negative regulator of autophagy.<sup>13</sup>

Metformin, a derivate of biguanide, is the first-line agent for treating T2DM patients. Metformin can lower blood sugar levels by inhibiting gluconeogenesis through adenosine monophosphate-activated protein kinase (AMPK) activation.<sup>14</sup> A previous study indicated that metformin treatment positively impacted skeletal muscle IR levels.<sup>15</sup> It also lowers fat mass, increases muscle mass which is atrophied due to obesity, and attenuates muscle cells oxidative stress in mice models.<sup>16,17</sup> However, some studies also reported that metformin might cause mitochondrial dysfunction and inhibit satellite cell proliferation, impeding mass muscle gain.<sup>18–20</sup> Microtubule-associated protein 1A/1B-light chain 3 (LC3) immunoblotting is commonly used to evaluate autophagy in intact muscle tissue. It is an autophagy-related gene that is essential for forming autophagosomes in muscle cells.<sup>21</sup>

Although several studies have examined the effects of Metformin on skeletal muscle in mice models, to our knowledge, the data regarding the effects of Metformin on human skeletal muscle is limited. Therefore, our present study aimed to investigate the effects of Metformin on autophagy through LC3 expression in human SkMC.

## METHODS

The present study was an experimental, *in vitro* laboratory research, with a posttest-only control group design, carried out at the laboratory of the physiology of Universitas Brawijaya. The T2DM human SkMC originated from Human Skeletal Muscle Myoblasts (HSMM) ABC-TC3957 T2DM cell line, which was obtained from T2DM treatment naïve patients, purchased from AcceGen Biotech®. The SkMC were grown in a 36-mm petri dish with glucose-rich DMEM (Dulbecco's Modified Eagle's medium containing 4.5 grams of glucose) and 20% Fetal Bovine Serum (FBS) and 1% antibiotic solution (penicillin-streptomycin-glutamine, PSG), incubated at 37°C with 5% CO<sub>2</sub> for three days. To fully differentiate the myotubes,

dishes with a density of 65 – 75% cells were cultured in DMEM with 2% Horse Serum and 1% PSG solution, incubated at 37°C with 5% CO<sub>2</sub> until the cell density reached 80% for a day. The media was changed every day during the fusion process, and the process was monitored with a contrast microscope using a 10 – 20 times magnification. Fully differentiated myotubes were randomized using the simple random sampling technique into control and treatment groups. The treatment group was given Metformin in three different doses (1 mM, 2 mM, and 3 mM), with 4 replications in each group, and then incubated for 2 days before the further examination.<sup>15,22,23</sup>

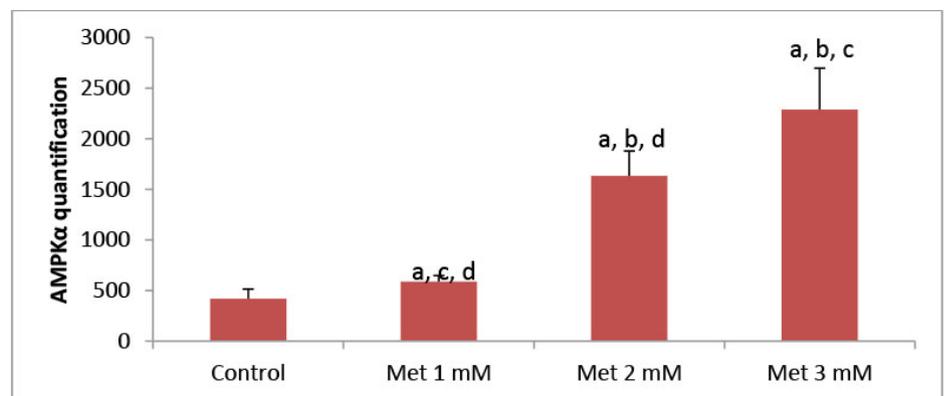
All myotubes were lysed, and then the lysates were rinsed with G-agarose for 20 minutes and incubated with a specific antibody overnight at 4°C. Protein G particles were precipitated for an hour at 4°C and then washed 4 times with lysis buffer solution before being inserted into polyacrylamide gel. After being inserted into 15% SDS-polyacrylamide gel, electrophoresis was performed on all samples. The nitrocellulose membrane was blocked with Tris buffer solution and then incubated overnight at 4°C with antibodies: anti-AMPK $\alpha$ 2 (Ser173) and anti-LC3. Anti-AMPK $\alpha$ 2 (Ser173) was diluted 1:2000.<sup>24</sup> The nitrocellulose membrane was then rinsed 3 times using PBS-T solution and incubated for an hour with

Horseshradish Peroxidase (HRP) secondary conjugated antibody.<sup>25,26</sup> Immunoblotting assay of AMPK $\alpha$  and LC3 was performed using ECL according to the manufacturer protocol based on the previous study.<sup>27,28</sup> The quantitative expression of AMPK $\alpha$  and LC3 were measured at baseline, after 24-hour, 48-hour, and 72-hour.

The collected data are presented as the mean  $\pm$  standard error (SE). Analysis using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was performed to detect a significant difference between groups. Kruskal-Wallis test was used instead for data that were not normally distributed. Differences were considered statistically significant at  $p < 0.05$ . Analysis using linear regression was performed to determine the correlation between the dose of metformin treatment, the timing of observation, and the expression of AMPK $\alpha$  and LC3. The analysis was performed using SPSS for Windows version 22.

## RESULTS

Since Metformin is a drug that acts through AMPK activation, the treatment group expressed AMPK $\alpha$  higher than the control group in this present study. This observation became more evident after 72-hour of metformin administration which displayed a significant difference in AMPK $\alpha$  levels from the control



**Figure 1.** Effects of Metformin on AMPK $\alpha$  expression. Immunoblotting quantification (mean  $\pm$  SE) was measured at 72-hour after Metformin was administered. Analysis of variance (ANOVA) followed by Tukey's post-hoc test results: <sup>a</sup> $p < 0.05$  when compared to control group, <sup>b</sup> $p < 0.05$  when compared to Met 1 mM group, <sup>c</sup> $p < 0.05$  when compared to Met 2 mM group, <sup>d</sup> $p < 0.05$  when compared to Met 3 mM group. Met 1 mM: group treated with 1 mM of Metformin; Met 2 mM: group treated with 2 mM of Metformin; Met 3 mM: group treated with 3 mM of Metformin.

group and each increment of metformin concentration (Figure 1). The level of AMPK $\alpha$  expression at baseline, after 24-hour, and 48-hour of metformin administration are represented in Figures 2, 3, and 4. Figure 5 depicts the visualization of AMPK $\alpha$  expression under

the fluorescent microscope for 72-hour.

Treatment with Metformin significantly increased the levels of LC3 expression in T2DM human SkMC. The treatment group with the concentration of 3 mM displayed the highest expression of LC3 levels both at baseline and after 24 48-hour of metformin

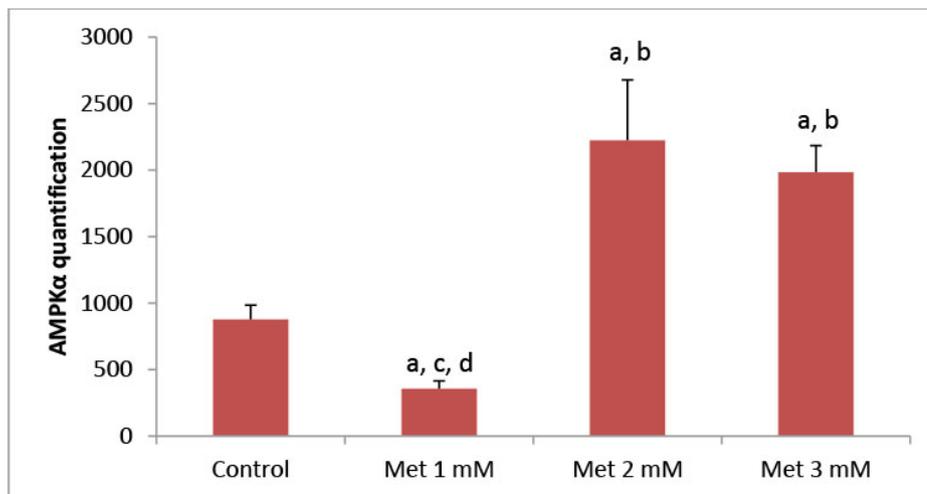
administration (Figures 6, 7, and 8). After 72-hour of metformin administration, LC3 expression in the group treated with 1 mM and 2 mM metformin raised significantly, displaying a statistically significant difference from the 3 mM group (Figure 9). Figure 10 represents the visualization of LC3 expression under the fluorescent microscope for 72-hour.

By using linear regression analysis, a significant correlation between metformin administration at increased concentrations and LC3 expression levels was found ( $p < 0.0001$ ). However, no significant correlation was established between the expression of AMPK $\alpha$  and LC3. These findings suggested that metformin-induced autophagy in T2DM human SkMC is somewhat AMPK $\alpha$  independent.

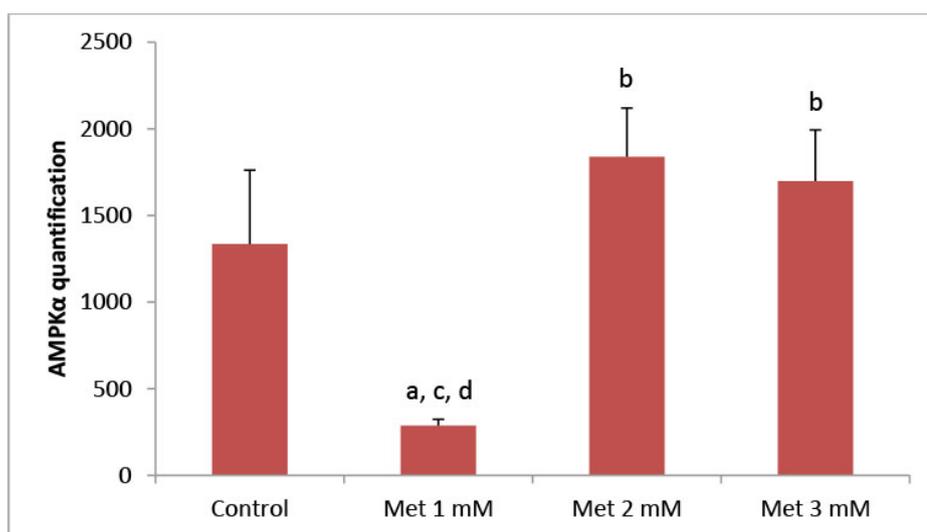
## DISCUSSION

Metformin is the first-line agent for treating hyperglycemia in T2DM patients. There is abundant evidence that the pharmacological action of Metformin is from the activation of AMPK and its downstream target. Considering AMPK as the main sensor of the cell's energy status, it also plays an important role in regulating autophagy. Previously, autophagy was presumed to promote sarcopenia of skeletal muscle. However, a line of evidence has accumulated, demonstrating that autophagy is important in skeletal muscle homeostasis. Skeletal muscle is an essential tissue in glucose metabolism and LC3 is widely used to monitor autophagy in skeletal muscle. Therefore, we are interested in investigating the effect of metformin administration on LC3 expression in T2DM human SkMC.

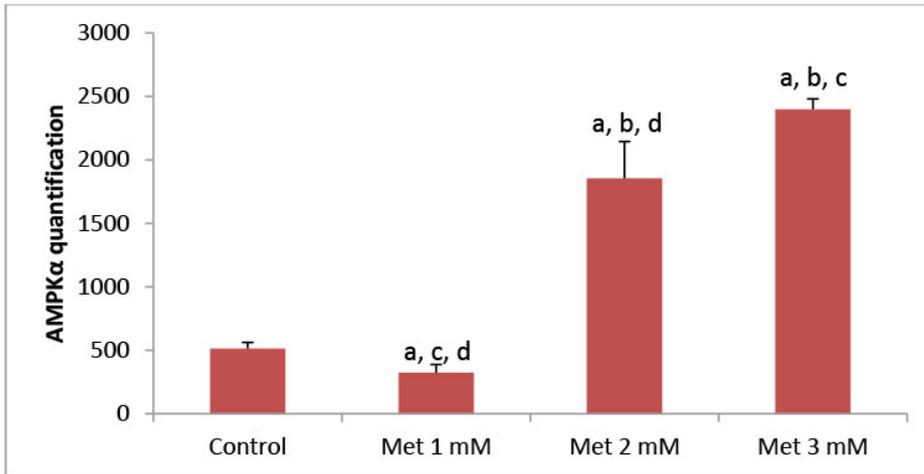
The process of autophagy is tightly regulated, involving numerous autophagy-related genes (ATG). In general, autophagy can be broken down into the following steps: initiation, vesicle (autophagosome) expansion, lysosome fusion, and degradation. As the sensor of cellular energy status, AMPK incites autophagy initiation, especially during starvation.<sup>29</sup> Numerous pharmacological agents are also known to target AMPK activation, directly and indirectly, for example, Metformin.<sup>30</sup> The ULK-1 complex activation is the central mechanism in autophagy initiation. Upon activation,



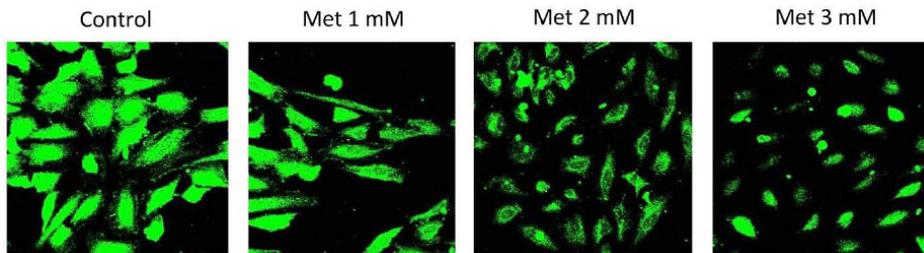
**Figure 2.** Effects of Metformin on AMPK $\alpha$  expression. Immunoblotting quantification (mean  $\pm$  SE) was measured at baseline. Analysis of variance (ANOVA) followed by Tukey's post-hoc test results: <sup>a</sup> $p < 0.05$  when compared to control group, <sup>b</sup> $p < 0.05$  when compared to Met 1 mM group, <sup>c</sup> $p < 0.05$  when compared to Met 2 mM group, <sup>d</sup> $p < 0.05$  when compared to Met 3 mM group. Met 1 mM: group treated with 1 mM of Metformin; Met 2 mM: group treated with 2 mM of Metformin; Met 3 mM: group treated with 3 mM of Metformin.



**Figure 3.** Effects of Metformin on AMPK $\alpha$  expression. Immunoblotting quantification (mean  $\pm$  SE) was measured at 24-hour after Metformin was administered. Analysis of variance (ANOVA) followed by Tukey's post-hoc test results: <sup>a</sup> $p < 0.05$  when compared to control group, <sup>b</sup> $p < 0.05$  when compared to Met 1 mM group, <sup>c</sup> $p < 0.05$  when compared to Met 2 mM group, <sup>d</sup> $p < 0.05$  when compared to Met 3 mM group. Met 1 mM: group treated with 1 mM of Metformin; Met 2 mM: group treated with 2 mM of Metformin; Met 3 mM: group treated with 3 mM of Metformin.



**Figure 4.** Effects of Metformin on AMPK $\alpha$  expression. Immunoblotting quantification (mean  $\pm$  SE) measured at 48-hour after Metformin was administered. Analysis of variance (ANOVA) followed by Tukey's post-hoc test results: <sup>a</sup> $p < 0.05$  when compared to control group, <sup>b</sup> $p < 0.05$  when compared to Met 1 mM group, <sup>c</sup> $p < 0.05$  when compared to Met 2 mM group, <sup>d</sup> $p < 0.05$  when compared to Met 3 mM group. Met 1 mM: group treated with 1 mM of Metformin; Met 2 mM: group treated with 2 mM of Metformin; Met 3 mM: group treated with 3 mM of Metformin.



**Figure 5.** Representative images of AMPK $\alpha$  expression with immunofluorescence at 72-hour after Metformin was administered. Met 1 mM: group treated with 1 mM of Metformin; Met 2 mM: group treated with 2 mM of Metformin; Met 3 mM: group treated with 3 mM of Metformin.

the ULK-1 complex then assembles the autophagosome with the Beclin 1-Vps34 complex in the next step. The autophagosome formation is completed once the two ubiquitin-like conjugation systems, namely ATG12-ATG5-ATG16L and ATG8, form the lipid conjugated form of LC3. The autophagosome is then fused with the lysosome to form the autolysosome, followed by degradation of its content.<sup>31</sup>

The main result of our present study was the significant correlation between metformin administration and LC3 expression. Metformin raised LC3 expression levels in the T2DM human SkMC dose-dependent. This finding is

similar to the study by Kanamori H et al., which demonstrated an increase in LC3 and autophagic activity in cardiomyocytes treated with Metformin in the long term.<sup>32</sup> Those findings might be due to inhibition of p62 activity, which suppresses autophagy. During starvation, the increase in AMPK $\alpha$  and FoxO3 expression will be followed by increased LC3 activity.<sup>25</sup> In contrast, Nwadike C et al., stated that AMPK activation might inhibit early and late phases of autophagy.<sup>33</sup> Autophagy regulation in skeletal muscle is also dependent on FoxO3 activity. In a starved skeletal muscle model, deletion of FoxO3 activity will impede the LC3 lipidation process.<sup>34</sup> This finding is also backed by

the study of Sanchez AM et al., on AICAR treated muscle cells which removed AMPK $\alpha$  and FoxO3 activity, still resulting in LC3 expression due to AMPK $\alpha$  and FoxO3 residual activity.<sup>25</sup>

Our present study demonstrated that Metformin raised the expression of AMPK $\alpha$  levels in T2DM human SkMC. This finding is supported by the study of Li DJ et al., in which metformin administration with a dose of 2 mM *in vitro* for 48-hour raised AMPK $\alpha$  expression levels significantly in skeletal muscle cells.<sup>22</sup> The elevation of AMPK $\alpha$  activity is related to the increase of Thr-172 phosphorylation and Acetyl-CoA Carboxylase (ACC) inactivation.<sup>35,36</sup> This increase in AMPK $\alpha$  activity is independent of the cellular ADP/ATP ratio.<sup>35</sup> However, some *in vivo* studies reported different results. The studies by Suwa M et al., in humans and Kristensen JM et al., in mice reported that even though the increase in AMPK $\alpha$  expression and ACC phosphorylation can be observed within 5 – 6 hours after metformin administration, after 14 days, these findings were negligible.<sup>37,38</sup> Administration of Metformin with the dose of 0.4 mM resulted in a dramatic increase in AMPK $\alpha$  expression within 24-hour without inhibition in mTOR expression.<sup>39</sup> Queiroz EA et al., also reported no significant difference in the AMPK $\alpha$  expression after 10 mM metformin administration in breast cancer cells, although notable phosphorylation of AMPK $\alpha$  and Thr-172 were observed after 48 and 72 hours.<sup>40</sup>

The present study failed to demonstrate a significant correlation between AMPK $\alpha$  and LC3 expression in the SkMC in the context of autophagic activity. This finding, however, is in contrast with several previous studies on autophagy in which the majority stated that AMPK is associated with autophagic flux. Autophagy is a complex process involving an interconnected web of key regulators rather than a linear pathway. Many of these regulators can induce autophagy through different requirements depending on the type and length of induction signals.<sup>41</sup> Aside from the AMPK-dependent Regulation of autophagic degradation, an AMPK-independent pathway triggers Ca<sup>2+</sup>-mediated autophagy, involving the

PI(3)P-effector protein WIPI-1 and LC3.<sup>42</sup> A study by Tomic T et al., regarding the autophagic and apoptotic properties of Metformin on melanoma cells, revealed that the increase in LC3 activity occurred by either AMPK-dependent or AMPK-independent pathways.<sup>43</sup>

Our study has several limitations

to be acknowledged. First, the doses of Metformin used in this present study were relatively small (1 mM, 2 mM, and 3 mM). The effect of a higher dose ( $\geq 5$  mM) of Metformin on the autophagic activity of human skeletal muscle cells still needs further investigation. Second, the HSMC ABC-TC3957 T2DM cell

lines used in this study were derived from Caucasians. Therefore, our results should be interpreted carefully, and future studies with more T2DM muscle cell lines are required to validate these findings. Third, our study only observed some of the autophagy-related proteins. Other downstream protein targets of AMPK (FoxO3, ULK-1, MURF-1) and their relation to autophagy in T2DM muscle cells still need to be investigated.

## CONCLUSION

This present study has demonstrated that metformin administration on T2DM human SkMC resulted in increased autophagic activity, marked by increased LC3 expression in a dose-dependent manner. Our study could not establish the correlation between the increase in AMPK $\alpha$  expression and LC 3 expression, which suggests that increased autophagy in the skeletal muscle might have occurred in AMPK-independent pathways.

## ACKNOWLEDGMENTS

None.

## CONFLICT OF INTEREST

The authors declared no conflict of interests.

## ETHICS CONSIDERATION

This research was reviewed and approved by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Airlangga, Surabaya, with reference number: 51/EC/KEPK/FKUA/2021.

## FUNDING

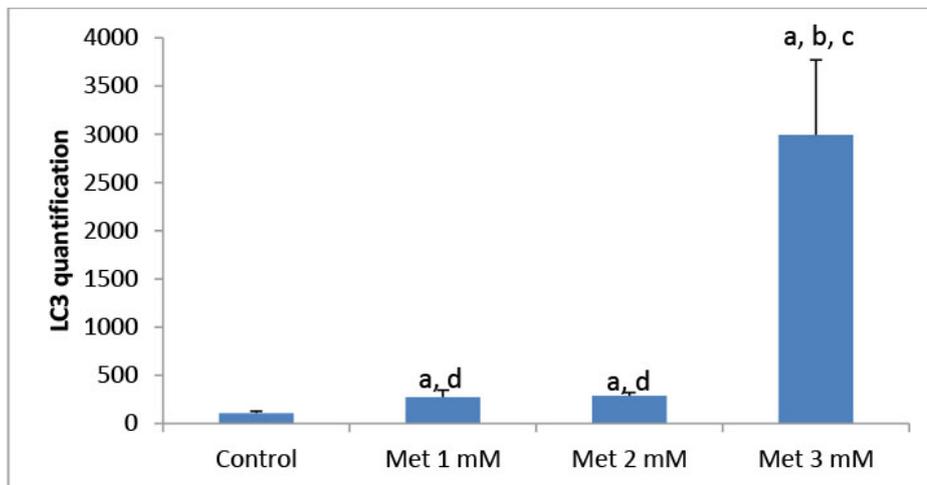
None.

## AUTHOR CONTRIBUTION

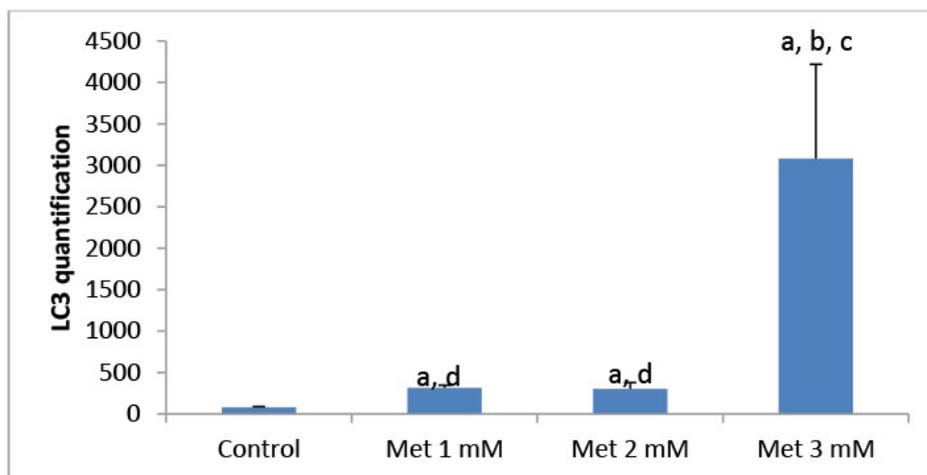
All authors contribute to the study from the conceptual framework, data acquisition, and data analysis until reporting the study results through publication.

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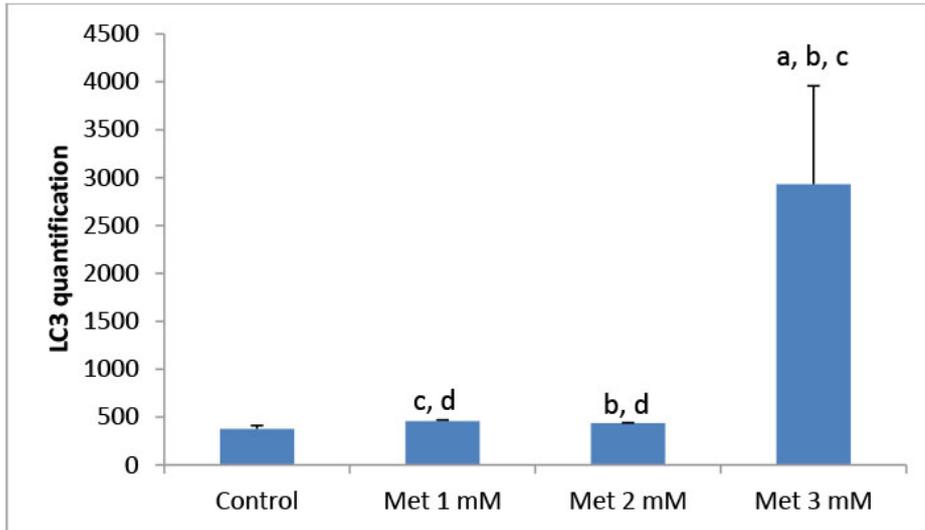


**Figure 6.** Effects of Metformin on LC3 expression. Immunoblotting quantification (mean  $\pm$  SE) was measured at baseline. Analysis of variance (ANOVA) followed by Tukey's post-hoc test results: <sup>a</sup> $p < 0.05$  when compared to control group, <sup>b</sup> $p < 0.05$  when compared to Met 1 mM group, <sup>c</sup> $p < 0.05$  when compared to Met 2 mM group, <sup>d</sup> $p < 0.05$  when compared to Met 3 mM group. Met 1 mM: group treated with 1 mM of Metformin; Met 2 mM: group treated with 2 mM of Metformin; Met 3 mM: group treated with 3 mM of Metformin.

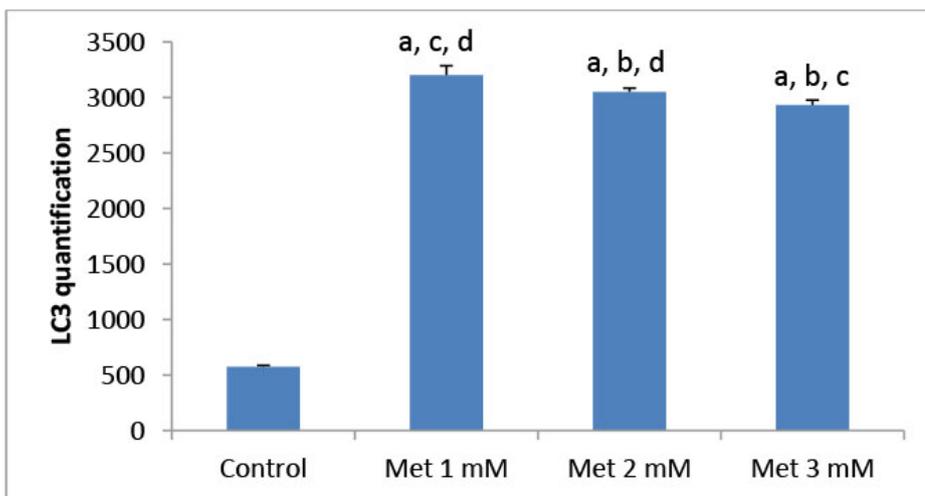


**Figure 7.** Effects of Metformin on LC3 expression. Immunoblotting quantification (mean  $\pm$  SE) was measured at 24-hour after Metformin was administered. Analysis of variance (ANOVA) followed by Tukey's post-hoc test results: <sup>a</sup> $p < 0.05$  when compared to control group, <sup>b</sup> $p < 0.05$  when compared to Met 1 mM group, <sup>c</sup> $p < 0.05$  when compared to Met 2 mM group, <sup>d</sup> $p < 0.05$  when compared to Met 3 mM group. Met 1 mM: group treated with 1 mM of Metformin; Met 2 mM: group treated with 2 mM of Metformin; Met 3 mM: group treated with 3 mM of Metformin.

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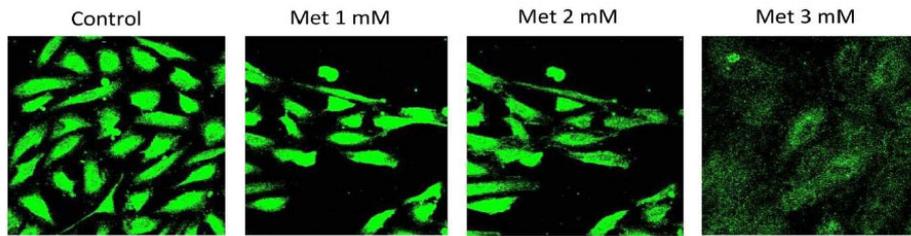


**Figure 8.** Effects of Metformin on LC3 expression. Immunoblotting quantification (mean ± SE) measured at 48-hour after Metformin was administered. Analysis of variance (ANOVA) followed by Tukey's post-hoc test results: <sup>a</sup> $p < 0.05$  when compared to control group, <sup>b</sup> $p < 0.05$  when compared to Met 1 mM group, <sup>c</sup> $p < 0.05$  when compared to Met 2 mM group, <sup>d</sup> $p < 0.05$  when compared to Met 3 mM group. Met 1 mM: group treated with 1 mM of Metformin; Met 2 mM: group treated with 2 mM of Metformin; Met 3 mM: group treated with 3 mM of Metformin.



**Figure 9.** Effects of Metformin on LC3 expression. Immunoblotting quantification (mean ± SE) was measured at 72-hour after Metformin was administered. Analysis of variance (ANOVA) followed by Tukey's post-hoc test results: <sup>a</sup> $p < 0.05$  when compared to control group, <sup>b</sup> $p < 0.05$  when compared to Met 1 mM group, <sup>c</sup> $p < 0.05$  when compared to Met 2 mM group, <sup>d</sup> $p < 0.05$  when compared to Met 3 mM group. Met 1 mM: group treated with 1 mM of Metformin; Met 2 mM: group treated with 2 mM of Metformin; Met 3 mM: group treated with 3 mM of Metformin.

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**Figure 10.** Representative images of LC3 expression with immunofluorescence at 72-hour after Metformin was administered. Met 1 mM: group treated with 1 mM of Metformin; Met 2 mM: group treated with 2 mM of Metformin; Met 3 mM: group treated with 3 mM of Metformin.

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