The effect of metformin on autophagy by LC3 expression in Type 2 Diabetes Mellitus

by Soebagijo Adi Soelistijo

Submission date: 14-Oct-2023 09:09PM (UTC+0800)
Submission ID: 2195460504
File name: n_on_autophagy_by_LC3_expression_in_Type_2_Diabetes_Mellitus.pdf (3.24M)
Word count: 5076
Character count: 27248

ORIGINAL ARTICLE

Bali Medical Journal (*Bali MedJ*) 2022, Volume 11, Number 1: 349-355
P-ISSN.2089-1180, E-ISSN: 2302-2914

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

Jongky Hendro Prajitno[1], Agung Pranoto[2*], Robert Dwitama Adiwinoto[3], Soebagijo Adi Soelistijo[2]

[1]Doctoral Program Student, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia;
[2]Division of Endocrinology, Diabetes, and Metabolism, Department of Internal Medicine, Dr. Soetomo Teaching Hospital, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia;
[3]Division of Endocrinology, Diabetes, and Metabolism, Department of Internal Medicine, Dr. Ramelan Naval Hospital, Surabaya, Indonesia;

*Corresponding author:
Agung Pranoto;
Division of Endocrinology, Diabetes, and Metabolism, Department of Internal Medicine, Dr. Soetomo Teaching Hospital, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia;
pranotoagung@yahoo.com

Received: 2022-02-13
Accepted: 2022-04-20
Published: 2022-04-30

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 AMPKα and LC3 was performed using electrochemiluminescence (ECL). The quantitative expression of AMPKα and LC3 were measured at baseline, after 24-hour, 48-hour, and 72-hour. Data were analyzed using SPSS version 22 for Windows.

Results: AMPKα and LC3 expression were higher in the treatment group compared to the control group. The levels of AMPKα 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.
Cite This Article: Prajitno, J.H., Pranoto, A., Adiwinoto, R.D., Soelistijo, S.A. 2022. The effect of metformin on autophagy by LC3 expression in Type 2 Diabetes Mellitus (T2DM) human skeletal muscle cell culture. *Bali Medical Journal* 11(1): 349-355.
DOI: 10.15562/bmj.v11i1.3203

INTRODUCTION

Skeletal muscle plays an important role in glucose metabolism.[1] 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.[2] Insulin triggers glucose uptake by muscle cells through the Glucose transporter (GLUT) and Sodium dependent glucose transporter-2 (SGLT-2).[3] 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.[4] Older patients with T2DM have a two-fold muscle mass loss compared to the elderly without T2DM.[5] Due to this important role in glucose utilization, loss of skeletal muscle mass (sarcopenia) will worsen insulin resistance (IR) in T2DM.[6]

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.[7] 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.[8]

Type 2 Diabetes Mellitus (T2DM) is a metabolic disorder that developed due to pancreatic islet β-cells' failure to sustain the hyperinsulinemia required to compensate for IR.[9,10] 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.[11] Previous study has demonstrated reduced autophagy response to metabolic stress in human muscle precursor cells from humans with T2DM.[12] Insulin has been linked to

inhibition of autophagy through activation of mammalian target of rapamycin (mTOR) which is a negative regulator of autophagy.[13]

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.[14] A previous study indicated that metformin treatment positively impacted skeletal muscle IR levels.[15] It also lowers fat mass, increases muscle mass which is atrophied due to obesity, and attenuates muscle cells oxidative stress in mice models.[16,17] However, some studies also reported that metformin might cause mitochondrial dysfunction and inhibit satellite cell proliferation, impeding mass muscle gain.[18–20] 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.[21]

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% CO2 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% CO2 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.[15,22,23]

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-AMPKα2 (Ser173) and anti-LC3. Anti-AMPKα2 (Ser173) was diluted 1: 2000.[24] The nitrocellulose membrane was then rinsed 3 times using PBS-T solution and incubated for an hour with Horseradish Peroxidase (HRP) secondary conjugated antibody.[25,26] Immunoblotting assay of AMPKα and LC3 was performed using ECL according to the manufacturer protocol based on the previous study.[27,28] The quantitative expression of AMPKα and LC3 were measured at baseline, after 24-hour, 48-hour, and 72-hour.

The collected data are presented as the mean ± 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 AMPKα 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 AMPKα 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 AMPKα levels from the control

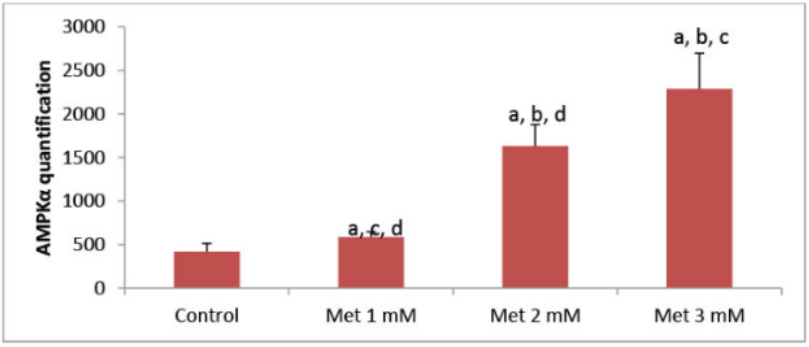

Figure 1. Effects of Metformin on AMPKα expression. Immunoblotting quantification (mean ± SE) was measured at 72-hour after Metformin was administered. Analysis of variance (ANOVA) followed by Tukey's post-hoc test results: [a]$p < 0.05$ when compared to control group, [b]$p < 0.05$ when compared to Met 1 mM group, [c]$p < 0.05$ when compared to Met 2 mM group, [d]$p < 0.05$ 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 AMPKα 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 AMPKα 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 AMPKα and LC3. These findings suggested that metformin-induced autophagy in T2DM human SkMC is somewhat AMPKα independent.

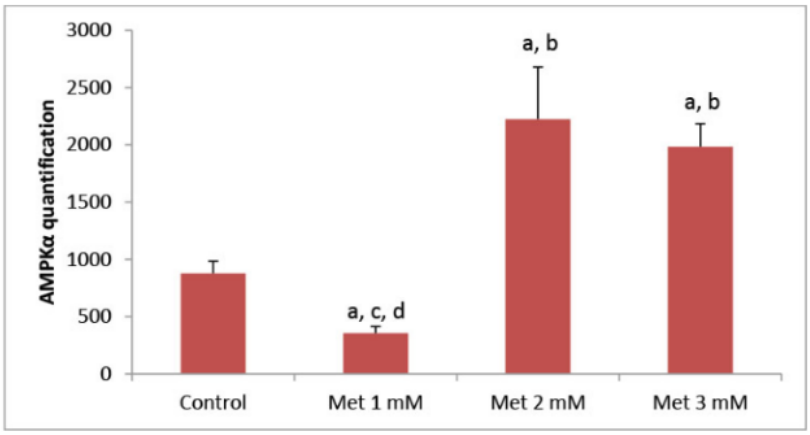

Figure 2. Effects of Metformin on AMPKα expression. Immunoblotting quantification (mean ± SE) was measured at baseline. Analysis of variance (ANOVA) followed by Tukey's post-hoc test results: $^{a}p < 0.05$ when compared to control group, $^{b}p < 0.05$ when compared to Met 1 mM group, $^{c}p < 0.05$ when compared to Met 2 mM group, $^{d}p < 0.05$ 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.

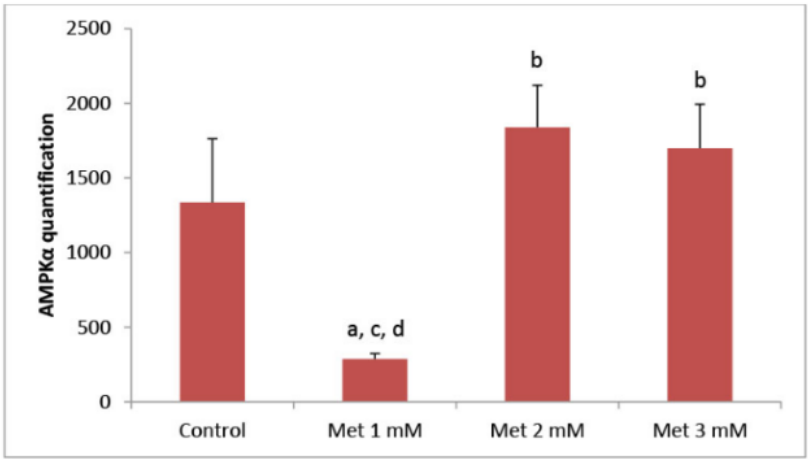

Figure 3. Effects of Metformin on AMPKα expression. Immunoblotting quantification (mean ± SE) was measured at 24-hour after Metformin was administered. Analysis of variance (ANOVA) followed by Tukey's post-hoc test results: $^{a}p < 0.05$ when compared to control group, $^{b}p < 0.05$ when compared to Met 1 mM group, $^{c}p < 0.05$ when compared to Met 2 mM group, $^{d}p < 0.05$ 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.

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 inT2DM 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.[29] Numerous pharmacological agents are also known to target AMPK activation, directly and indirectly, for example, Metformin.[30] The ULK-1 complex activation is the central mechanism in autophagy initiation. Upon activation,

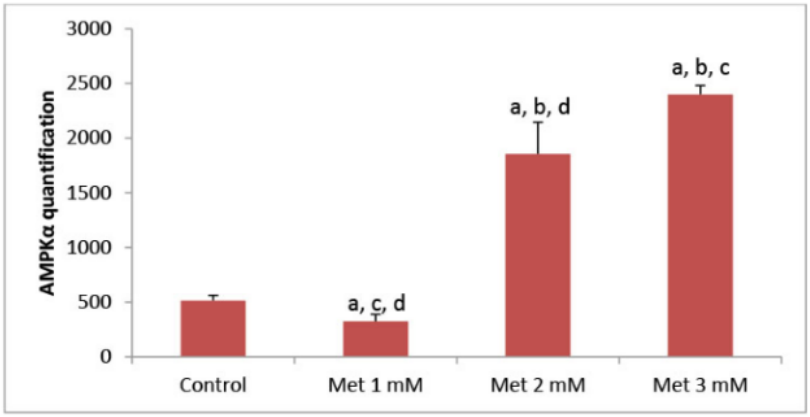

Figure 4. Effects of Metformin on AMPKα expression. Immunoblotting quantification (mean ± SE) measured at 48-hour after Metformin was administered. Analysis of variance (ANOVA) followed by Tukey's post-hoc test results: $^{a}p < 0.05$ when compared to control group, $^{b}p < 0.05$ when compared to Met 1 mM group, $^{c}p < 0.05$ when compared to Met 2 mM group, $^{d}p < 0.05$ 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.

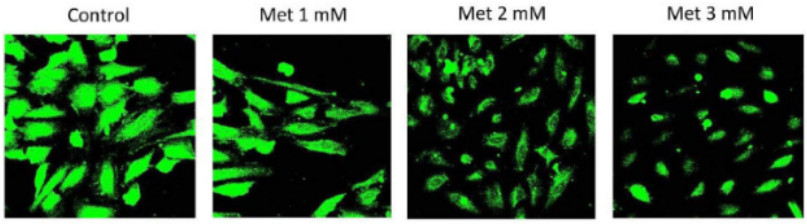

Figure 5. Representative images of AMPKα expression with immunofluorescence at 72-hour after Metformin was administered. Met 1 mM: group treated with 1 mM of Metformin; Met 2 mM: group treated with 2 mM of Metformin; Met 3 mM: group treated with 3 mM of Metformin.

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.[31]

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.[32] Those findings might be due to inhibition of p62 activity, which suppresses autophagy. During starvation, the increase in AMPKα and FoxO3 expression will be followed by increased LC3 activity.[25] In contrast, Nwadike C et al., stated that AMPK activation might inhibit early and late phases of autophagy.[33] 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.[34] This finding is also backed by the study of Sanchez AM et al., on AICAR treated muscle cells which removed AMPKα and FoxO3 activity, still resulting in LC3 expression due to AMPKα and FoxO3 residual activity.[25]

Our present study demonstrated that Metformin raised the expression of AMPKα 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 AMPKα expression levels significantly in skeletal muscle cells.[22] The elevation of AMPKα activity is related to the increase of Thr-172 phosphorylation and Acetyl-CoA Carboxylase (ACC) inactivation.[35,36] This increase in AMPKα activity is independent of the cellular ADP/ATP ratio.[35] 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 AMPKα expression and ACC phosphorylation can be observed within 5 – 6 hours after metformin administration, after 14 days, these findings were negligible.[37,38] Administration of Metformin with the dose of 0.4 mM resulted in a dramatic increase in AMPKα expression within 24-hour without inhibition in mTOR expression.[39] Queiroz EA et al., also reported no significant difference in the AMPKα expression after 10 mM metformin administration in breast cancer cells, although notable phosphorylation of AMPKα and Thr-172 were observed after 48 and 72 hours.[40]

The present study failed to demonstrate a significant correlation between AMPKα 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.[41] Aside from the AMPK-dependent Regulation of autophagic degradation, an AMPK-independent pathway triggers Ca^{2+}-mediated autophagy, involving the

PI(3)P-effector protein WIPI-1 and LC3.[42] 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.[43]

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 (≥ 5 mM) of Metformin on the autophagic activity of human skeletal muscle cells still needs further investigation. Second, the HSMM 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.

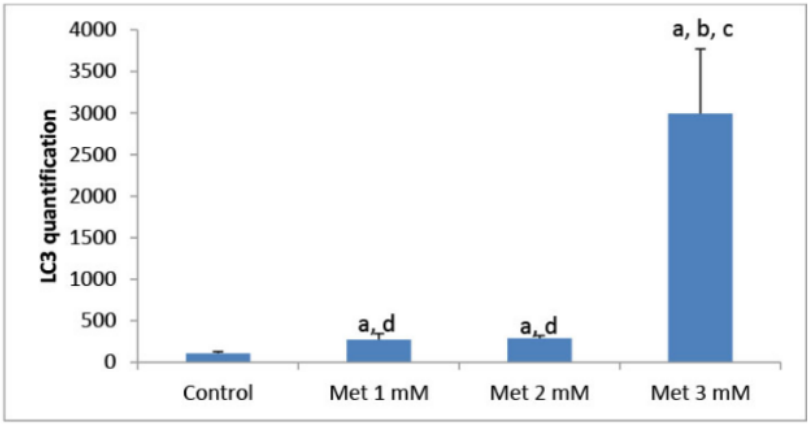

Figure 6. Effects of Metformin on LC3 expression. Immunoblotting quantification (mean ± SE) was measured at baseline. Analysis of variance (ANOVA) followed by Tukey's post-hoc test results: [a]$p < 0.05$ when compared to control group, [b]$p < 0.05$ when compared to Met 1 mM group, [c]$p < 0.05$ when compared to Met 2 mM group, [d]$p < 0.05$ 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.

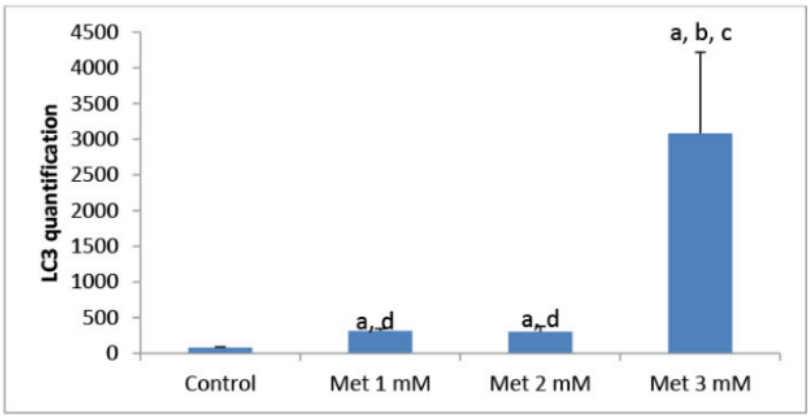

Figure 7. Effects of Metformin on LC3 expression. Immunoblotting quantification (mean ± SE) was measured at 24-hour after Metformin was administered. Analysis of variance (ANOVA) followed by Tukey's post-hoc test results: [a]$p < 0.05$ when compared to control group, [b]$p < 0.05$ when compared to Met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

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 AMPKα 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.

REFERENCES

1. Jaiswal N, Gavin MG, Quinn WJ 3rd, Luongo TS, Gelfer RG, Baur JA, et al. The role of skeletal muscle Akt in the regulation of muscle mass and glucose homeostasis. Mol Metab. 2019;28:1-13.

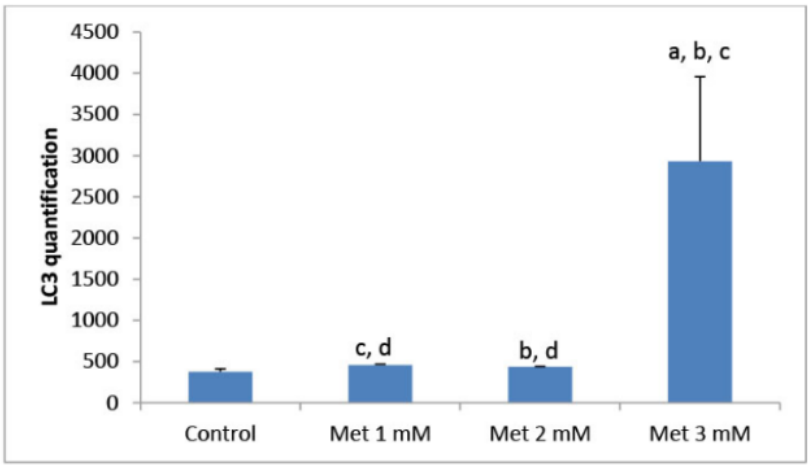

Figure 8. Effects of Metformin on LC3 expression. Immunoblotting quantification (mean ± SE) measured at 48-hour after Metformin was administered. Analysis of variance (ANOVA) followed by Tukey's post-hoc test results: $^{a}p < 0.05$ when compared to control group, $^{b}p < 0.05$ when compared to Met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

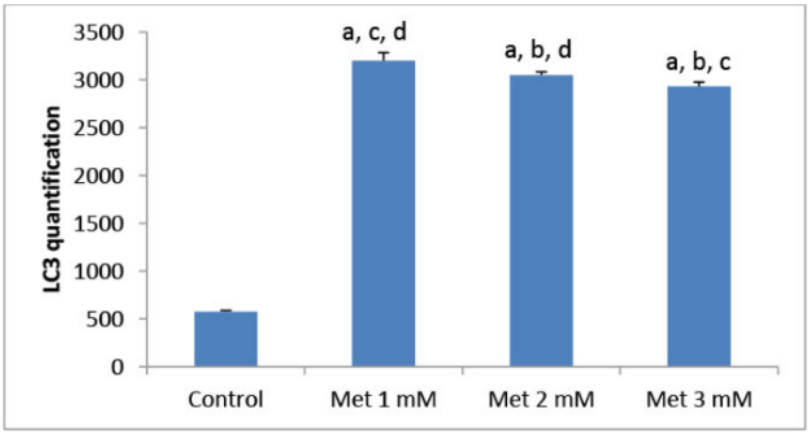

Figure 9. Effects of Metformin on LC3 expression. Immunoblotting quantification (mean ± SE) was measured at 72-hour after Metformin was administered. Analysis of variance (ANOVA) followed by Tukey's post-hoc test results: $^{a}p < 0.05$ when compared to control group, $^{b}p < 0.05$ when compared to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

2. Bawadi H, Alkhatib D, Abu-Hijleh H, Alalwani J, Majed L, Shi Z. Muscle Strength and Glycaemic Control among Patients with Type 2 Diabetes. *Nutrients*. 2020;12(3):771.
3. Evans PL, McMillin SL, Weyrauch LA, Witczak CA. Regulation of Skeletal Muscle Glucose Transport and Glucose Metabolism by Exercise Training. *Nutrients*. 2019;11(10):2432.
4. Sinacore DR, Gulve EA. The role of skeletal muscle in glucose transport, glucose homeostasis, and insulin resistance: implications for physical therapy. *Phys Ther*. 1993;73(12):878-891.
5. Park SW, Goodpaster BH, Lee JS, Kuller LH, Boudreau R, de Rekeneire N, et al. Excessive loss of skeletal muscle mass in older adults with type 2 diabetes. Diabetes Care. 2009 Nov;32(11):1993-7.
6. Abidin Öztürk ZA, Türkbeyler İH, Demir Z, Bilici M, Kepekçi Y. The effect of blood glucose regulation on sarcopenia parameters in obese and diabetic patients. *Turk J Phys Med Rehabil*. 2017;64(1):72-79.
7. Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. *Cell*. 2011;147(4):728-741.
8. Jiao J, Demontis F. Skeletal muscle autophagy and its role in sarcopenia and organismal aging. *Curr Opin Pharmacol*. 2017;34:1-6.
9. Prentki M, Nolan CJ. Islet beta cell failure in type 2 diabetes. *J Clin Invest*. 2006;116(7):1802-1812.
10. Kahn SE, Cooper ME, Del Prato S. Pathophysiology and treatment of type 2 diabetes: perspectives on the past, present, and future. *Lancet*. 2014;383(9922):1068-1083.
11. Dudani S, Poodury S, Mangalesh S. Study of neutrophil-lymphocyte ratio (NLR) in recent onset type 2 diabetes mellitus. Bali Medical Journal. 2021;10(1):11-16.
12. Henriksen TI, Wigge LV, Nielsen J, Pedersen BK, Sandri M, Scheele C. Dysregulated autophagy in muscle precursor cells from humans with type 2 diabetes. *Sci Rep*. 2019;9(1):8169.
13. Settembre C, Di Malta C, Polito VA, Garcia Arencibia M, Vetrini F, Erdin S, et al. TFEB links autophagy to lysosomal biogenesis. Science. 2011;332(6036):1429-33.
14. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest. 2001;108(8):1167-74.
15. Yuan H, Hu Y, Zhu Y, Zhang Y, Luo C, Li Z, et al. Metformin ameliorates high uric acid-induced insulin resistance in skeletal muscle cells. Mol Cell Endocrinol. 2017;443:138-145.
16. Hasan MM, Shalaby SM, El-Gendy J, Abdelghany EMA. Beneficial effects of metformin on muscle atrophy induced by obesity in rats. *J Cell Biochem*. 2019;120(4):5677-5686.
17. Diniz Vilela D, Gomes Peixoto L, Teixeira RR, Belele Baptista N, Carvalho Caixeta D, Vieira de Souza A, et al. The Role of Metformin in Controlling Oxidative Stress in Muscle of Diabetic Rats. *Oxid Med Cell Longev*. 2016;2016:6978625.
18. Wessels B, Ciapaite J, van den Broek NM, Nicolay K, Prompers JJ. Metformin impairs mitochondrial function in skeletal muscle of both lean and diabetic rats in a dose-dependent manner. *PLoS One*. 2014;9(6):e100525.
19. Mulyani WRW, Sanjiwani MID, Sandra, Prabawa IPY, Lestari AAW, Wihandani DM, et al. Chaperone-Based Therapeutic Target Innovation: Heat Shock Protein 70 (HSP70) for Type 2 Diabetes Mellitus. Diabetes Metab Syndr Obes. 2020;13:559-568.
20. Walton RG, Dungan CM, Long DE, Tuggle SC, Kosmac K, Peck BD, et al. Metformin blunts

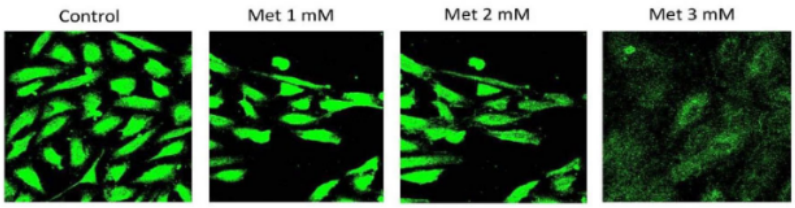

Figure 10. Representative images of LC3 expression with immunofluorescence at 72-hour after Metformin was administered. Met 1 mM: group treated with 1 mM of Metformin; Met 2 mM: group treated with 2 mM of Metformin; Met 3 mM: group treated with 3 mM of Metformin.

muscle hypertrophy in response to progressive resistance exercise training in older adults: A randomized, double-blind, placebo-controlled, multicenter trial: The MASTERS trial. Aging Cell. 2019;18(6):e13039.

21. Fritzen AM, Madsen AB, Kleinert M, Treebak JT, Lundsgaard AM, Jensen TE, et al. Regulation of autophagy in human skeletal muscle: effects of exercise, exercise training and insulin stimulation. J Physiol. 2016 Feb 1;594(3):745-61.
22. Li DJ, Huang F, Lu WJ, Jiang GJ, Deng YP, Shen FM. Metformin promotes irisin release from murine skeletal muscle independently of AMP-activated protein kinase activation. *Acta Physiol (Oxf)*. 2015;213(3):711-721.
23. Jadhav KS, Dungan CM, Williamson DL. Metformin limits ceramide-induced senescence in C2C12 myoblasts. *Mech Ageing Dev*. 2013;134(11-12):548-559.
24. Brown AE, Dibnah B, Fisher E, Newton JL, Walker M. Pharmacological activation of AMPK and glucose uptake in cultured human skeletal muscle cells from patients with ME/CFS. *Biosci Rep*. 2018;38(3):BSR20180242.
25. Sanchez AM, Csibi A, Raibon A, Cornille K, Gay S, Bernardi H, et al. AMPK promotes skeletal muscle autophagy through activation of forkhead FoxO3a and interaction with Ulk1. J Cell Biochem. 2012;113(2):695-710.
26. Clavel S, Siffroi-Fernandez S, Coldefy AS, Boulukos K, Pisani DF, Dérijard B. Regulation of the intracellular localization of Foxo3a by stress-activated protein kinase signaling pathways in skeletal muscle cells. *Mol Cell Biol*. 2010;30(2):470-480.
27. Thomson DM. The Role of AMPK in the Regulation of Skeletal Muscle Size, Hypertrophy, and Regeneration. *Int J Mol Sci*. 2018;19(10):3125.
28. Mizushima N, Yoshimori T. How to interpret LC3 immunoblotting. *Autophagy*. 2007;3(6):542-545.
29. Xia Q, Huang X, Huang J, Zheng Y, March ME, Li J, et al. The Role of Autophagy in Skeletal Muscle Diseases. Front Physiol. 2021;12:638983.
30. Coughlan KA, Valentine RJ, Ruderman NB, Saha AK. AMPK activation: a therapeutic target for type 2 diabetes?. *Diabetes Metab Syndr Obes*. 2014;7:241-253.
31. Sebastián D, Zorzano A. Self-Eating for Muscle Fitness: Autophagy in the Control of Energy Metabolism. *Dev Cell*. 2020;54(2):268-281.
32. Kanamori H, Naruse G, Yoshida A, Minatoguchi S, Watanabe T, Kawaguchi T, et al. Metformin Enhances Autophagy and Provides Cardioprotection in δ-Sarcoglycan Deficiency-Induced Dilated Cardiomyopathy. Circ Heart Fail. 2019;12(4):e005418.
33. Nwadike C, Williamson LE, Gallagher LE, Guan JL, Chan EYW. AMPK Inhibits ULK1-Dependent Autophagosome Formation and Lysosomal Acidification via Distinct Mechanisms. *Mol Cell Biol*. 2018;38(10):e00023-18.
34. Milan G, Romanello V, Pescatore F, Armani A, Paik JH, Frasson L, et al. Regulation of autophagy and the ubiquitin-proteasome system by the FoxO transcriptional network during muscle atrophy. Nat Commun. 2015;6:6670.
35. Hawley SA, Gadalla AE, Olsen GS, Hardie DG. The antidiabetic drug metformin activates the AMP-activated protein kinase cascade via an adenine nucleotide-independent mechanism. *Diabetes*. 2002;51(8):2420-2425.
36. Musi N, Hirshman MF, Nygren J, Svanfeldt M, Bavenholm P, Rooyackers O, et al. Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes. Diabetes. 2002;51(7):2074-81.
37. Suwa M, Egashira T, Nakano H, Sasaki H, Kumagai S. Metformin increases the PGC-1alpha protein and oxidative enzyme activities possibly via AMPK phosphorylation in skeletal muscle in vivo. *J Appl Physiol (1985)*. 2006;101(6):1685-1692.
38. Kristensen JM, Treebak JT, Schjerling P, Goodyear L, Wojtaszewski JF. Two weeks of metformin treatment induces AMPK-dependent enhancement of insulin-stimulated glucose uptake in mouse soleus muscle. *Am J Physiol Endocrinol Metab*. 2014;306(10):E1099-E1109.
39. Langone F, Cannata S, Fuoco C, Lettieri Barbato D, Testa S, Nardozza AP, et al. Metformin protects skeletal muscle from cardiotoxin induced degeneration. PLoS One. 2014;9(12):e114018.
40. Queiroz EA, Puukila S, Eichler R, Sampaio SC, Forsyth HL, Lees SJ, et al. Metformin induces apoptosis and cell cycle arrest mediated by oxidative stress, AMPK and FOXO3a in MCF-7 breast cancer cells. PLoS One. 2014;9(5):e98207.
41. Corona Velazquez AF, Jackson WT. So Many Roads: the Multifaceted Regulation of Autophagy Induction. *Mol Cell Biol*. 2018;38(21):e00303-18.
42. Grotemeier A, Alers S, Pfisterer SG, Paasch F, Daubrawa M, Dieterle A, et al. AMPK-independent induction of autophagy by cytosolic Ca2+ increase. Cell Signal. 2010;22(6):914-25.
43. Tomic T, Botton T, Cerezo M, Robert G, Luciano F, Puissant A, et al. Metformin inhibits melanoma development through autophagy and apoptosis mechanisms. Cell Death Dis. 2011;2(9):e199.

The effect of metformin on autophagy by LC3 expression in Type 2 Diabetes Mellitus

ORIGINALITY REPORT

17%	12%	14%	7%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	docserv.uni-duesseldorf.de Internet Source	1%
2	tesisenred.net Internet Source	1%
3	f1000research.com Internet Source	<1%
4	www.acarindex.com Internet Source	<1%
5	www.oncotarget.com Internet Source	<1%
6	Submitted to Butler University Student Paper	<1%
7	benthamscience.com Internet Source	<1%
8	encyclopedia.pub Internet Source	<1%
9	prism.ucalgary.ca Internet Source	<1%

10	www.researchgate.net Internet Source	<1%
11	Füllgrabe, Jens, Daniel J. Klionsky, and Bertrand Joseph. "The return of the nucleus: transcriptional and epigenetic control of autophagy", Nature Reviews Molecular Cell Biology, 2013. Publication	<1%
12	Submitted to SDM Universitas Gadjah Mada Student Paper	<1%
13	wjgnet.com Internet Source	<1%
14	www.emjreviews.com Internet Source	<1%
15	Javier A. Menendez. "mTOR-regulated senescence and autophagy during reprogramming of somatic cells to pluripotency: A roadmap from energy metabolism to stem cell renewal and aging", Cell Cycle, 11/01/2011 Publication	<1%
16	Submitted to Universiti Kebangsaan Malaysia Student Paper	<1%
17	William W. Winder, David M. Thomson. "Cellular energy sensing and signaling by	<1%

	AMP-activated protein kinase", Cell Biochemistry and Biophysics, 2007 Publication	
18	cris.maastrichtuniversity.nl Internet Source	<1 %
19	Martin J.T. Kalsbeek, Samantha E.C. Wolff, Nikita L. Korpel, Susanne E. la Fleur et al. "The impact of antidiabetic treatment on human hypothalamic infundibular neurons and microglia", JCI Insight, 2020 Publication	<1 %
20	cyberleninka.org Internet Source	<1 %
21	ijphs.iaescore.com Internet Source	<1 %
22	journal.ipb.ac.id Internet Source	<1 %
23	repository.um.edu.my Internet Source	<1 %
24	curis.ku.dk Internet Source	<1 %
25	Chung, Chung. "The Effects of Calorie Restriction on Autophagy: Role on Aging Intervention", Nutrients, 2019 Publication	<1 %

26	Henrik Oster. "Getting hot about diabetes—Repeated heat exposure improves glucose regulation and insulin sensitivity", Acta Physiologica, 2020 Publication	<1 %
27	ecite.utas.edu.au Internet Source	<1 %
28	www.em-consulte.com Internet Source	<1 %
29	www.spandidos-publications.com Internet Source	<1 %
30	Submitted to The University of Manchester Student Paper	<1 %
31	asp.journals.umz.ac.ir Internet Source	<1 %
32	bmcpublichealth.biomedcentral.com Internet Source	<1 %
33	Submitted to De Montfort University Student Paper	<1 %
34	E Laane. "Cell death induced by dexamethasone in lymphoid leukemia is mediated through initiation of autophagy", Cell Death and Differentiation, 04/24/2009 Publication	<1 %
35	cytojournal.com Internet Source	

		<1 %
36	www.preprints.org Internet Source	<1 %
37	W W Mukhammad, C Kembangsari, Y A A J Santoso, I R Mukti, H B P Putra, R P Adiwinoto, R D Adiwinoto. "M18. Three-year cardiovascular outcome of SGLT-2 Inhibitor in patient with Type 2 Diabetes Mellitus: a Meta-Analysis of Randomized Control Trial", European Heart Journal Supplements, 2021 Publication	<1 %
38	coek.info Internet Source	<1 %
39	e-journal.unair.ac.id Internet Source	<1 %
40	pjmhsonline.com Internet Source	<1 %
41	www.scribd.com Internet Source	<1 %
42	www.todaysdietitian.com Internet Source	<1 %
43	Nguyen, H G, J C Yang, H-J Kung, X-B Shi, D Tilki, P N Lara, R W DeVere White, A C Gao, and C P Evans. "Targeting autophagy overcomes Enzalutamide resistance in	<1 %

castration-resistant prostate cancer cells and improves therapeutic response in a xenograft model", Oncogene, 2014.
Publication

44	clinicalepigeneticsjournal.biomedcentral.com Internet Source	<1 %
45	wiadlek.pl Internet Source	<1 %
46	www.jbc.org Internet Source	<1 %
47	www.tandfonline.com Internet Source	<1 %
48	Hiromitsu Kanamori, Genki Naruse, Akihiro Yoshida, Shingo Minatoguchi et al. "Metformin Enhances Autophagy and Provides Cardioprotection in δ-Sarcoglycan Deficiency-Induced Dilated Cardiomyopathy", Circulation: Heart Failure, 2019 Publication	<1 %
49	Matthew P. Bubak, Shivani N. Mann, Agnieszka K. Borowik, Atul Pranay et al. "17α-estradiol Alleviates High-Fat Diet-Induced Inflammatory and Metabolic Dysfunction in Skeletal Muscle of Male and Female Mice", Cold Spring Harbor Laboratory, 2023 Publication	<1 %

50	Yang Liu, Yajie Hu, Shize Li. "Protein O-GlcNAcylation in Metabolic Modulation of Skeletal Muscle: A Bright but Long Way to Go", Metabolites, 2022 Publication	<1 %
51	discovery.ucl.ac.uk Internet Source	<1 %
52	www.thno.org Internet Source	<1 %
53	Emma Barroso, Marta Montori-Grau, Walter Wahli, Xavier Palomer, Manuel Vázquez-Carrera. "Striking a gut–liver balance for the antidiabetic effects of metformin", Trends in Pharmacological Sciences, 2023 Publication	<1 %
54	Cecconi, F.. "The Role of Autophagy in Mammalian Development: Cell Makeover Rather than Cell Death", Developmental Cell, 20080916 Publication	<1 %
55	Geissler, Catherine, Powers, Hilary. "Human Nutrition", Human Nutrition, 2023 Publication	<1 %
56	Jan Henriksson. "Muscle fuel selection: effect of exercise and training", Proceedings of the Nutrition Society, 2007 Publication	<1 %

57	Kelin Li, Minghui Wang, Ruxia Wang, Xiaojuan Wang, Hongchao Jiao, Jingpeng Zhao, Yunlei Zhou, Haifang Li, Hai Lin. "Hydrogen Sulfide Regulates Glucose Uptake in Skeletal Muscles via S-Sulfhydration of AMPK in Muscle Fiber Type-Dependent Way", The Journal of Nutrition, 2023 Publication	<1 %
58	L. J. Rubin, L. Magliola, X. Feng, A. W. Jones, C. C. Hale. "Metabolic activation of AMP kinase in vascular smooth muscle", Journal of Applied Physiology, 2005 Publication	<1 %
59	Ryan N. Montalvo, Brittany R. Counts, James A. Carson. "Understanding sex differences in the regulation of cancer-induced muscle wasting", Current Opinion in Supportive and Palliative Care, 2018 Publication	<1 %
60	Salminen, Antero, Anu Kauppinen, and Kai Kaarniranta. "Inflammaging Signaling in Health Span and Life Span Regulation", Inflammation Advancing Age and Nutrition, 2014. Publication	<1 %
61	Submitted to University of Nizwa Student Paper	<1 %

Exclude quotes	Off	Exclude matches	Off
Exclude bibliography	On		

The effect of metformin on autophagy by LC3 expression in Type 2 Diabetes Mellitus

GRADEMARK REPORT

FINAL GRADE

/100

GENERAL COMMENTS

PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5

PAGE 6

PAGE 7