**Response to the reviewers**

**Manuscript ID:** JTRM-D-25-05906

**Manuscript Title:** Nonhistone lactylation: A hub for tumour metabolic reprogramming and epigenetic regulation

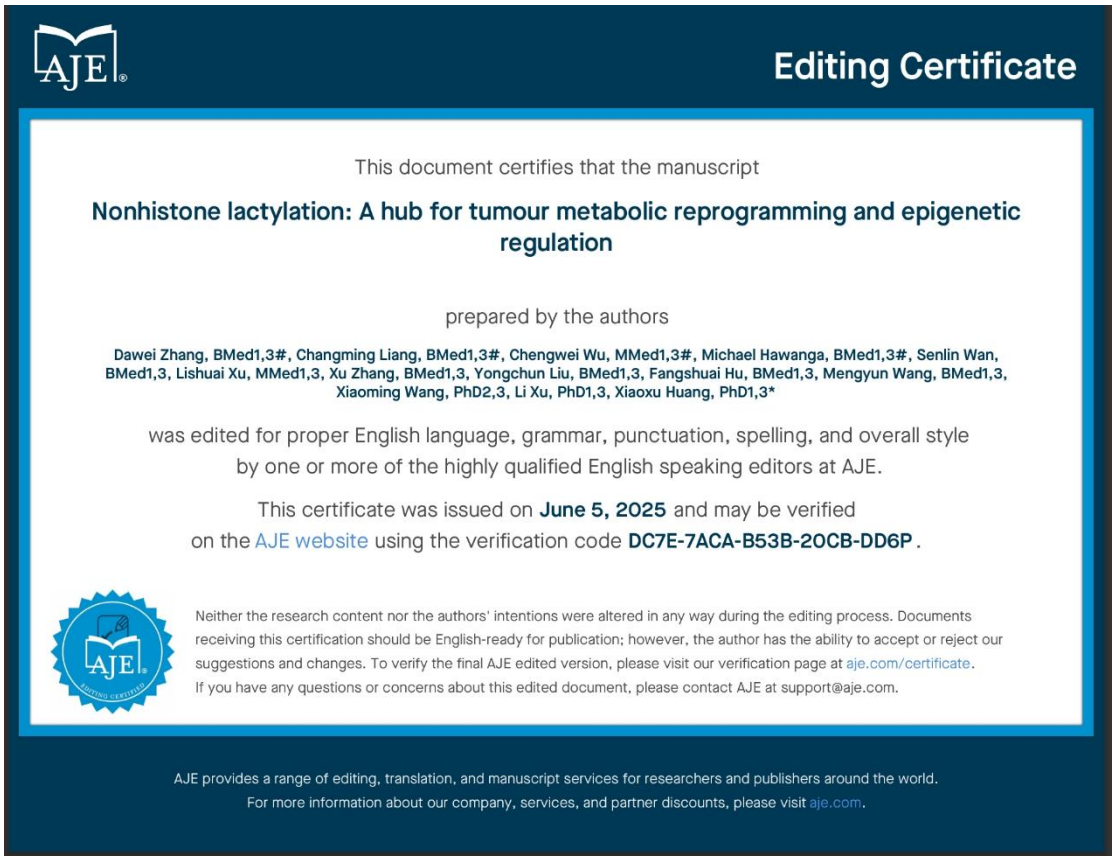
Dear Reviewers:

Special thanks to you for your comments. We have tried our best to improve the manuscript and made some changes in the manuscript according to your suggestions; all changes made to the text are in blue font. We appreciate for your warm work earnestly; and hope that the correction will meet with approval.

Reviewer #1:

1. The language needs revision by a native speaker and a manuscript with tracked editing records as a Suppl.

*Author response: Thank you very much for your great efforts on our manuscript. I have touched up the manuscript on the American Journal Experts website and submitted the manuscript with editorial records as a supplement. An editing certificate is provided below：*



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**Nonhistone lactylation: A hub for tumour metabolic reprogramming and epigenetic regulation**

prepared by the authors

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2. For a better understanding of Introduction and Discussion, please cite the following literature in appropriate site (PMID). Please inform the reference no. of your cited references clearly.

Neoplasms remain the main killer worldwide [37080257, 34326874, 37541791, 32984321].

*Author response: Thank you for your valuable suggestions, we have carefully reviewed the literature you recommended and cited them in the Introduction and Discussion with reference numbers (10), (67), (104), (103), and in blue in the manuscript.*

3. The discussion is not in-depth, limitation is lack, and the clinical implications shall also be added.

*Author response: Thank you for your insightful feedback. We have substantially revised the Discussion section to comprehensively address these points:1. Elaboration of Clinical Implications: In the future, single-cell sequencing, spatial metabolomics and dynamic imaging technologies should be combined to analyse the spatial and temporal distributions of lactylation in the tumour microenvironment and to clarify their specific roles in primary foci, metastatic foci, and the process of treatment resistance (17, 36). Development of precision-targeted therapies. The development of highly selective inhibitors or agonists targeting key enzymes (e.g., AARS1, the SIRT family) and metabolic pathways (e.g., LDH, MCTs) involved in lactylation is central to clinical translation (13, 33, 103). Integration of immunotherapy with metabolic regulation-related modifications shapes the immunosuppressive microenvironment by modulating PD-L1 stability, Treg cell function and macrophage polarization. Dual-targeting strategies can be designed in the future: immune checkpoint inhibitors, for example, anti-PD-1/PD-L1 drugs, can be combined with lactate transport inhibitors (MCT1/4 antagonists) to break the immunosuppressive barrier (14). Combined metabolic–epigenetic interventions increase T-cell infiltration and killing activity by inhibiting the HIF-1α or glycolytic pathway and decreasing lactate levels while targeting lactylation-related enzymes (60). Breakthroughs in drug resistance mechanisms and individualized diagnosis and treatment have led to the establishment*

*of drug resistance marker profiles and the development of dynamic monitoring technologies to guide clinical drug selection and real-time assessment of therapeutic response and drug resistance risk (15, 38, 104).*2. *Addition of Limitations Section: Unmet clinical needs and challenges of off-target effects and safety: Lactation modifications are widely involved in normal physiological processes and are needed to ensure the precision of targeted therapies. Metabolic plasticity: Tumour cells may escape treatment through compensatory pathways, requiring the design of combined multipathway interventions. Clinical translation barriers: Promotion of phase I/II clinical trials targeting lactate modifications to clarify pharmacokinetic profiles and optimal dosing strategies.* *Changes in the manuscript are highlighted in blue font. The revisions are listed below:*

The study of nonhistone lactylation as a key hub linking tumour metabolic reprogramming and epigenetic regulation has provided novel perspectives and potential targets for tumour therapy. Although current studies have revealed the central role of lactation modification in tumorigenesis, drug resistance, and immune escape, future in-depth explorations in the following directions are still needed to promote the translation of basic research to clinical applications: analysis of the mechanisms regulating spatiotemporal dynamics and spatial and temporal heterogeneity of lactylation may determine their functional differences at different stages of tumour development. In the future, single-cell sequencing, spatial metabolomics and dynamic imaging technologies should be combined to analyse the

spatial and temporal distributions of lactylation in the tumour microenvironment and to clarify their specific roles in primary foci, metastatic foci, and the process of treatment resistance (17, 36). Development of precision-targeted therapies. The development of highly selective inhibitors or agonists targeting key enzymes (e.g., AARS1, the SIRT family) and metabolic pathways (e.g., LDH, MCTs) involved in lactylation is central to clinical translation (13, 33, 103). Integration of immunotherapy with metabolic regulation-related modifications shapes the immunosuppressive microenvironment by modulating PD-L1 stability, Treg cell function and macrophage polarization. Dual-targeting strategies can be designed in the future: immune checkpoint inhibitors, for example, anti-PD-1/PD-L1 drugs, can be combined with lactate transport inhibitors (MCT1/4 antagonists) to break the immunosuppressive barrier (14). Combined metabolic–epigenetic interventions increase T-cell infiltration and killing activity by inhibiting the HIF-1α or glycolytic pathway and decreasing lactate levels while targeting lactylation-related enzymes (60). Breakthroughs in drug resistance mechanisms and individualized diagnosis and treatment have led to the establishment of drug resistance marker profiles and the development of dynamic monitoring technologies to guide clinical drug selection and real-time assessment of therapeutic response and drug resistance risk (15, 38, 104). Integrating advanced technologies such as nanotechnology will be pivotal. Nanosensors could enable real-time monitoring of spatiotemporal lactylation dynamics within the complex tumour microenvironment. Furthermore, nanocarriers offer promising platforms for the targeted delivery of novel lactylation inhibitors (e.g.,

targeting AARS1, SIRTs, or specific lactylation sites) or synergistic therapeutic agents (e.g., glycolysis inhibitors combined with immunotherapy drugs), enhancing tumour specificity and overcoming biological barriers while minimizing systemic toxicity (105, 106). Unmet clinical needs and challenges of off-target effects and safety: Lactation modifications are widely involved in normal physiological processes and are needed to ensure the precision of targeted therapies. Metabolic plasticity: Tumour cells may escape treatment through compensatory pathways, requiring the design of combined multipathway interventions. Clinical translation barriers: Promotion of phase I/II clinical trials targeting lactate modifications to clarify pharmacokinetic profiles and optimal dosing strategies.

In conclusion, nonhistone lactylation research is moving from mechanism exploration to clinical translation. Breakthroughs in this field will not only provide new weapons for tumour treatment but also may reshape the metabolism–epigenetic–immunomodulation combined therapeutic paradigm and ultimately realize the transition from "treating the symptom" to "treating the root cause."

Reviewer #2:

Comments:

This paper has several issues that make it unsuitable for publication currently, and the manuscript needs to address the following concerns before it can possibly be accepted:

1. Regarding introduction:

The introduction failed to construct the foundation of the necessity of the review. The first paragraph, the only "introduction" paragraph, is too blurry to sacrifice its scientific logic. Line 62 "In addition…thousands of other metabolites." Line 63 "Recent research has revealed that these small molecules, which are produced during cellular metabolism, can covalently modify proteins and serve as substrates for epigenetic regulation." What are the purposes of these sentences? Why would authors mention unrelated metabolites? Authors are blending concepts into the introduction by mentioning them in separate unrelated sentences without aligning them into the review framework. The following sentences on PTM (Line 65) cannot establish a relationship with the prior context. In line 73, the authors jump to nonhistone acetylation from histone modification by stating "Research has shown that not only histones but also nonhistone proteins such as p53, PD-L1 can be modified by lactate, altering their functions." This paragraph needs surgery to subdivide into several paragraphs. Suggestions would be 1) start with lactate, a general introduction of its role in human metabolism, and its evolving roles in cancers; 2) continue with PTMs; 3) NonPTM lactylation and its potential roles. Consider adding details on histone lactylation, especially on why it is not "a hub for tumour metabolic reprogramming and epigenetic regulation".

*Author response: Thank you for your constructive feedback. We sincerely appreciate your detailed suggestions to improve the logical flow and scientific rigor of the introduction. We have thoroughly restructured this section into three logical and clear paragraphs based on your suggestions. The revisions in the manuscript are highlighted in blue font. The revisions are listed below:*

Lactate plays an important role in human physiological metabolism as an end product of glycolysis (1). Under normal physiological conditions, when tissues and organs are in a hypoxic environment, such as muscle tissue during strenuous exercise, cells rapidly convert glucose to lactate through the glycolytic pathway and produce a small amount of ATP to satisfy the energy needs of the cell (2, 3). Moreover, lactate can be used as a raw material for gluconeogenesis and is converted to glucose in the liver, kidneys and other organs to maintain stable blood glucose levels (4). In addition, lactate is involved in the regulation of acid–base balance and the maintenance of homeostasis in the internal environment (1). Under physiological conditions, lactate is involved as a signalling molecule in the regulation of neural activity, lipolysis and energy metabolism (5-7). However, in the TME, there is a significant shift in the function of lactate. Tumour cells tend to produce large amounts of lactate from glycolysis even under aerobic conditions, known as the "Warburg effect". These lactate molecules not only provide biosynthetic raw materials and energy for tumour cells but also promote tumour cell invasion, inhibit immune cell function, and induce

angiogenesis by acidifying the microenvironment, creating favourable conditions for malignant tumour progression (8-10).

Protein posttranslational modifications (PTMs) are important ways for cells to precisely regulate protein function. Cells alter the structure, activity, localization and interaction of proteins by adding or removing chemical groups, such as those involved in phosphorylation, acetylation, and methylation, to specific amino acid residues of proteins, which in turn regulate physiological processes such as cell proliferation, differentiation, and apoptosis. Abnormal alterations in PTMs play a key role in tumour development (11).

Nonhistone lactylation is a novel PTM mediated by lactate that regulates protein function by covalently binding lysine residues (12). Nonhistone lactylation involves a variety of key proteins, such as p53, PD-L1, and MRE11, which directly affect tumorigenesis, immune escape, and drug resistance (13-15). Histone lactylation occurs mainly on lysine residues of histones H3 and H4, which can alter chromatin conformation and affect gene transcription. However, it is not central to tumour metabolic reprogramming and epigenetic regulation because its modification sites are limited, and tumour regulation involves a complex network of DNA methylation, other modifications of histone proteins, synergistic interactions between transcription factors, and metabolites that weaken the centrality of histone lactonization (16-18).

2. Regarding Lactate metabolism in cancer cells:

Line 98, "Typically…enabling swift ATP production" Authors established an unspecified detail on glycolysis and its swift ATP production. Do authors have reliable sources on numbers? Why does glycolysis produce only 2 ATP molecules but at a "faster pace"? Is it because Line 102 "cancer cells enhance glycolysis through various mechanisms? If so, revise line 98 as this is not directly evidenced.

*Author response: Thank you for raising this key issue. We have revised and improved the text and added supporting references. The modification of the text section has been indicated in the manuscript and marked in blue font. The revisions are listed below:*

During the process of glycolysis, glucose is converted into pyruvate. The majority (85 %) of pyruvate is converted into lactate by LDH, even in the presence of sufficient oxygen, which is a manifestation of the Warburg effect. Only a small fraction (5 %) of pyruvate enters the mitochondria for the TCA cycle, where ATP is produced (22). Although the glycolytic pathway can only produce 2 molecules of ATP, rapid ATP production can be achieved in cancer cells because the reaction process does not require the involvement of mitochondria and the activity levels of key enzymes are up-regulated. Moreover, cancer cells can further increase their glycolytic flux through

the upregulation of glucose transporter proteins and the activation of signalling pathways to satisfy the energy demands of rapid proliferation (25, 26).

3. Regarding Mechanisms of tumour regulation by nonhistone lactylation modifications:

Line 169 "In this study, we reveal RCC2 has a novel role in recruiting RNA-binding proteins and regulating RNA stability." Please specify which study and/or how authors reveal the role of RCC2. If it is detailed in the following sentence, please consider rephrasing the sentence. A similar problem is in Line 253, "in this study, we found that…" Line 300 "Here we identify…"Line 227, 350 "we identify…". Line 221, "we found that…" Line 242. Line 283.Line 306

*Author response: Thank you for highlighting this critical issue. We systematically revised all "in this study", "we found", and similar phrases, and have highlighted the revisions in blue in the manuscript. The revisions are listed below:*

Line 169: Fang Lin's group reported that RCC2 has a novel role in recruiting RNA-binding proteins and regulating RNA stability. Specifically, KAT2A mediates RCC2 lactylation at K124, which triggers the recruitment of free SERBP1 via the N-terminal domain of RCC2 and leads to the formation of the initial SERBP1-MAD2L1 complex. SERBP1 directly binds and stabilizes MAD2L1 mRNA, ultimately upregulating MAD2L1 protein expression (50).

Line 253: According to Fan's team, FDX1, which is responsible for reducing $Cu^{2+}$ to a more toxic form, $Cu^{1+}$, is dramatically increased under high-level copper conditions; high copper content promotes METTL16-K229 lactylation, and then lactylated METTL16 increases FDX1 mRNA and protein levels via $m6^A$ modification of FDX1 mRNA, which ultimately induces DLAT lipolylation and cuproptosis in gastric carcinoma (GC) (32).

Line 300: You's team revealed that lysine-specific demethylase 1 (LSD1) lactylation, which is induced by the reaccumulation of lactate in both human and murine BRAFi/MEKi-resistant melanoma cells, selectively drives survival via epigenetic reprogramming.

Line 227: DCBLD1 is an emulsification substrate, and its major emulsification site is located at K172. DCBLD1 overexpression inhibits G6PD autophagic degradation, activates the PPP, and promotes cervical cancer progression (56, 57).

Line 350: Thus, lactate is an essential small molecule that reinforces Treg cells in the TME through lactylation (61).

Line 221: Additionally, lactate increased DCBLD1 expression, activating the PPP to facilitate the proliferation and metastasis of cervical cancer cells.

Line 242: Zhou's team reported that the key elements of the Hippo pathway, YAP and TEAD1, possess conserved sites for lactylation.

Line 283: In addition to MRE11 and NBS1, another important HR protein, RAD51, is lactonated at K73 and K40 (15, 43, 68).

Line 306: Lactylated LSD1 codirects gene transcription with FosL1 to repress ferroptosis via interference with transferrin receptor protein 1 (TFRC)-mediated iron uptake (69).

4. Regarding in-text reference:

Authors shall check the manuscript whether statements have clear reference support. If so, in-text citations shall be added. Details are provided below.

Other Comments:

a. Check the format of author prefix and affiliation in the author list to align with the JCM publication requirement.

*Author response: Thank you for highlighting this important issue. We have carefully read the JCM guidelines for authors and have revised the format of author prefix and*

*affiliation in the author list to fully comply with the requirements of the journal.*

b. Add references if have. Line 65, 71, 75, 95, 100,102, 114, 117, 118, 173, 185, 203, 223, 226, 227, 261, 263, 279, 284, 308, 336, 339, 344, 347, 369. These listed sentences were core sentences in each section without clear sources.

*Author response: Thank you for your careful review. We sincerely appreciate your suggestions for strengthening the evidence base of the manuscript. We have carefully added relevant references to all labeled lines to ensure that each key statement is supported by authoritative literature. Reference numbers in the manuscript are marked in blue.*

c. Renew Figure legend 1-3. Figure 1 "MCT1 transfers L-lactate into the cell." Figure 2 "There is active glycolysis…" Figure 3 "…plays a role in (e.g….)"Consider modifying these legends as they are not typical/professional.

*Author response: Thank you for your valuable comments on how to improve the professionalism of the figure legends. In an effort to align with academic standards, we have thoroughly revised all of the figure legends, and the revisions in the manuscript are highlighted in blue font; below are the specific changes made to each figure legends:*

Figure 1: Molecular mechanism of lactylation mediated by L-lactate.

MCT1 transfers L-lactate into the cell. The glycolysis byproduct pyruvate undergoes LDHA-mediated conversion to produce L-lactate. L-lactate undergoes lacty-CoA synthetase (GTPSCS/ACSS2) actions to synthesize lactyl-CoA, which is involved in histone and nonhistone Kl-la by the actions of related writers(P300/CBP/KAT8/TIP60/HB01). In addition, L-lactate and ATP form lactate-AMP and PPi in response to AARS1/2. Lactate-AMP is covalently linked to lysines on target proteins. Kla is de-lactylated in the presence of erasers (HDAC1-3/SIRT).

Figure 2: Nonhistone lactylation affects tumour development through different regulatory mechanisms

Tumour cells produce large amounts of lactate through the Warburg effect, Lactate further affects the regulation of gene expression, metabolic regulation, signalling pathways, induction of cellular cuproptosis and cellular autophagy through nonhistone lactylation, ultimately promoting tumorigenesis. In addition, lactylation modulates tumour drug resistance through DNA damage repair, inhibition of ferroptosis, M6A modification and antioxidant mechanisms. Meanwhile, lactate promotes tumour immune escape by regulating gene expression, enhancing PD-L1 stability and immune cell regulation. OXA and 2-DG inhibit the Warburg effect, whereas AARS1 inhibitors and Huperzine inhibit lactylation -related enzymes.

Figure 3: Association of nonhistone lactylation with diverse diseases and cancers. Nonhistone lactylation is associated with pathologies such as cardiovascular disease (CVD), systemic lupus erythematosus (SLE), arthritis, Alzheimer's disease (AD), retinopathy and stress. At the same time, nonhistone lactylation affects the development and progression of a variety of cancers, including colorectal cancer (CRC), hepatocellular carcinoma (HCC), gastric cancer (GC), non-small cell lung cancer (NSCLC), oropharyngeal squamous cell carcinoma (OSCC), and glioblastoma (GBM).

c. Figure 3. Provide notes on abbreviations if they are not shown in the text.

*Author response: Thank you for your valuable feedback. We have refined the full list of abbreviations and their full names in the manuscript to ensure clarity for the reader.*

**Abbreviations**

PTM: Protein post-translational modification

TME: Tumour microenvironment

MCT: Monocarborxylat transporter

HIF-1α: Hypoxia-inducible factor-1 α

LDH: Lactate dehydrogenase

OSCC: Oral squamous cell carcinoma

GSCs: Glioma stem cells

LCSCs: Liver cancer stem cells

HCC: Hepatocellular carcinoma

MGO: Methylglyoxal

GBC: Gallbladder cancer

NCL: Nucleolin

ICCA: Intrahepatic cholangiocarcinoma

PC: Prostate cancer

G6PD: Glucose-6-phosphate dehydrogenase

GC: Gastric carcinoma

GBM: Glioblastoma

BRAFi: BRAF inhibitor

MEKi: MEK inhibitor

LSD1: Lysine-specific demethylase 1

PCK2: Phosphoenolpyruvate carboxykinase 2

2-DG: 2-Deoxy-D-glucose

α-CHC: α-Cyano-4-hydroxycinnamate

CVD: Cardiovascular disease

SLE: Systemic lupus erythematosus

AD: Alzheimer's disease

CRC: Colorectal Cancer

NSCLC: Nonsmall cell lung cancer