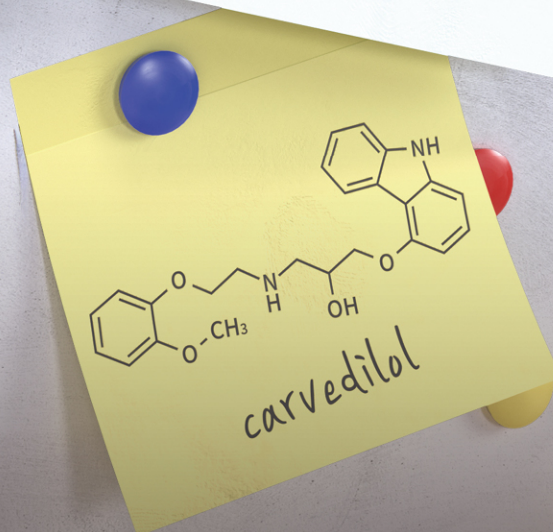
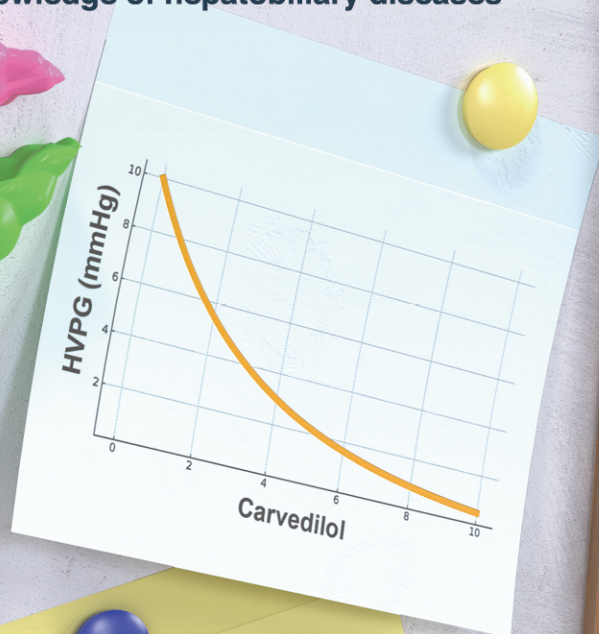


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Correspondence to editorial on “DNA methylome analysis reveals epigenetic alteration of complement genes in advanced metabolic dysfunction-associated steatotic liver disease”

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Dear Editor,

We appreciate the opportunity to respond to the comments by Dr. DiStefano and Dr. Gerhard on our recent publication.^{1,2} Their editorial provides a comprehensive overview of our analysis of epigenetic alterations in complement genes associated with metabolic dysfunction-associated steatotic liver disease (MASLD). We would like to address three key issues raised in their commentary:

Do changes in DNA methylation of complement genes directly contribute to MASLD pathogenesis or are they a consequence of progressive liver damage and metabolic disturbances characteristic of the disease?

DNA methylation plays a crucial role in regulating gene expression and maintaining cellular identity. However, abnormal methylation patterns can disrupt cellular homeostasis, and contribute to disease progression.³ In our study, we provide robust evidence of epigenetic alterations in complement genes linked to the progression of MASLD. By conducting an integrative analysis of DNA methylome and transcriptome data from liver biopsies of MASLD patients,

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we discovered a consistent pattern: hypermethylation and downregulation of six genes (*C1R*, *C1S*, *C3*, *C6*, *C4BPA*, and *SERPING1*), alongside hypomethylation and upregulation of three genes (*C5AR1*, *C7*, and *CD59*). The methylation patterns of nine selected complement genes showed correlation with the histological severity of MASLD, encompassing liver fibrosis, NAS, liver steatosis, lobular inflammation, and ballooning. Particularly, the methylation status of *C1R* showed a robust inverse correlation with its expression in metabolic dysfunction–associated steatohepatitis (MASH) ($R=-0.58$) and metabolic dysfunction-associated steatotic liver (MASL) ($R=-0.47$), whereas no discernible correlation was observed in control samples ($R=0.013$). Similarly, for *C5AR1*, a strong inverse correlation was found in MASH ($R=-0.67$), with relatively weaker correlations in MASL ($R=-0.22$) and controls ($R=-0.15$), suggesting that DNA methylation changes within nine complement genes increase with increasing severity of MASLD, with consequent effects on expression.

As MASLD progresses, liver injury and fibrosis may disrupt the liver microenvironment, potentially inducing epigenetic changes, including altered DNA methylation patterns.⁴ These changes could shift the expression of complement genes, which might represent a secondary response to ongoing liver damage rather than a primary causative factor of the disease. Additionally, the metabolic disturbances commonly observed in MASLD, such as insulin resistance, hyperglycemia, and oxidative stress, can drive shifts in DNA methylation patterns. This may create a feedback loop in which the disease induces further epigenetic modifications, amplifying its own progression and complicating disease management.

DNA methyltransferases and TET enzymes play crucial roles in regulating DNA methylation and demethylation, often targeting specific genomic regions such as repetitive elements, centromeric regions, promoters, and enhancers. However, these enzymes inherently lack precise target specificity and are guided by factors beyond minor sequence variations near the CpG sites.⁵ Metabolic intermediates serve as critical regulators of DNA methylation and demethylation, linking metabolic homeostasis with epigen-

etic modifications. Disruptions in metabolic balance can lead to altered cell-specific methylation patterns, contributing to disease development, including MASLD.⁶ The methyl group for DNA methylation is derived from S-adenosylmethionine, which is synthesized through the methionine cycle involving essential nutrients such as methionine, folate, choline, betaine, and vitamins B2, B6, and B12. DNA demethylation rates are similarly sensitive to metabolic fluctuations, with TET enzymes utilizing α -ketoglutarate, a TCA cycle intermediate, to remove methyl groups. Metabolites like fumarate and succinate can inhibit TET enzyme activity by competing with α -ketoglutarate, highlighting how cellular metabolic conditions influence the equilibrium between DNA methylation and demethylation.

The role of complement gene methylation at specific CpG sites in the development of MASLD could be further validated using CRISPR-based editing of DNA methylation sites, known as CRISPR on-off systems.⁷ Due to the cellular heterogeneity within the liver, MASLD-associated DNA methylation alterations are likely to vary significantly across different hepatic cell types. Recent advances in single-cell transcriptomics and epigenomics offer promising opportunities to enhance our understanding of the pathogenesis of MASLD and its associated complications, potentially leading to more targeted therapeutic approaches.

Taken together, the relationship between DNA methylation of complement genes and MASLD is likely bidirectional. Epigenetic changes may both contribute to disease development and arise as a consequence of the disease, creating a complex interplay that needs further investigation to fully understand.

Three complement genes (*C7*, *C5AR1*, and *CD59*) were predominantly expressed in non-hepatocyte cells and exhibited hypomethylation and upregulation in MASLD samples. Given the liver's diverse cellular composition, further single-cell analysis is necessary to clarify these findings

The liver contains various non-parenchymal and immune

Abbreviations:

MASH, metabolic dysfunction–associated steatohepatitis; MASL, metabolic dysfunction-associated steatotic liver; MASLD, metabolic dysfunction-associated steatotic liver disease

cells, including macrophages, stellate cells, and lymphoid cells. In MASLD specimens, *C7*, *C5AR1*, and *CD59* were primarily expressed in non-parenchymal cells and were found to be hypomethylated and upregulated. MacParland et al.⁸ used single-cell RNA sequencing to profile the human liver's cellular landscape, analyzing 8,444 parenchymal and non-parenchymal cells isolated from fresh hepatic tissue from five human livers. Their study identified *C7*, a terminal component of the complement cascade, as enriched in periportal liver sinusoidal endothelial cells, while *C5AR1* was primarily expressed in CD68+ macrophages. *C5aR1* plays a significant role in proinflammatory processes, regulating the expression of TNF- α , IL-1 β , and other proinflammatory factors, with NLRP3 being a central component of this pathway.⁹ Interestingly, *C5aR1* deficiency has been shown to protect against diet-induced MASH by regulating inflammatory responses and promoting efferocytosis of hepatic macrophages.⁹

An increasing amount of single-cell data, including spatial multi-omics of MASLD specimens, will provide detailed insights into the roles of these genes during MASLD progression. Advances in DNA methylation profiling technologies, such as single-cell combinatorial indexing for methylation analysis (sci-MET)¹⁰ and single-nucleus methyl-3C sequencing (sn-m3C-seq),¹¹ present promising tools for further elucidating complement gene methylation at the single-cell level.

Understanding the relationship between methylation, mRNA expression, and protein levels of the nine complement genes in the liver, particularly in MASLD, is of significant interest

Methylation status can regulate gene expression, influencing protein levels and thereby affecting liver health and disease progression. Hepatocytes, the primary cells responsible for extracellular complement synthesis, express six complement genes (*C1R*, *C1S*, *C3*, *C6*, *C4BPA*, and *SERPING1*) that show increased methylation in MASLD. The proteins encoded by these genes, once synthesized and activated, may play a crucial role in preserving hepatocyte function and regenerative potential. In contrast, three complement genes (*C7*, *C5AR1*, and *CD59*), mainly expressed in non-parenchymal cells, were found to be hypomethylated and upregulated in MASLD samples. Epigene-

tic modifications in these genes may impair the innate immune response, potentially contributing to liver injury and fibrosis. Immunohistochemical analysis of these complement proteins in MASLD specimens could reveal their protein expression patterns. Advanced technologies like multiplexed imaging, single-cell spatial transcriptomics, and ultra-sensitive proteomics could provide deeper insights into the causal relationships between complement components and MASLD progression.

Our study on the epigenetic modifications in complement genes linked to MASLD suggests a complex, bidirectional relationship between DNA methylation and MASLD, indicating that epigenetic changes may both contribute to and arise from MASLD progression. The intricate interplay among DNA methylation, gene expression, and metabolic disturbances highlights the multifaceted nature of MASLD pathogenesis, warranting further investigation using advanced technologies such as CRISPR-based epigenome editing, single-cell epigenomics, and spatial multi-omics. A deeper understanding of these mechanisms could ultimately guide the development of targeted therapeutic strategies for MASLD, enhancing patient outcomes.

Authors' contribution

A. M., H.-J. K., W. K., and M. K. conceived and designed the study. M. K. wrote the manuscript. W.K. revised the manuscript. All authors read and approved the final manuscript.

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Conflicts of Interest

The authors have no conflicts to disclose.

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