

## LETTERS TO THE EDITOR

Readers may submit letters to the editor concerning articles that appeared in Cellular and Molecular Gastroenterology and Hepatology within one month of publication. Detailed guidelines regarding the content are included in the Instructions to Authors.

### Necroptosis in Nonalcoholic Steatohepatitis



Dear Editor:

One fundamental step along the sequence from benign liver steatosis toward progressive steatohepatitis is the occurrence of hepatocyte cell death, currently classified as apoptosis. However, necroptosis, governed by the kinase receptor-interacting protein 3 (RIP3), has emerged as an alternative programmed cell-death pathway.<sup>1</sup> Petra Hirsova and Gregory J. Gores, who have made many important contributions to the field of cell death in the liver, published an interesting article in *Cellular and Molecular Gastroenterology and Hepatology*<sup>2</sup> in which they provided a comprehensive overview on the role of programmed cell death pathways in NASH. We would like to take the chance to discuss their statement that “it is unlikely that necroptosis contributes to liver injury and inflammation in NASH” because we believe that it might be too early to exclude this important new cell death pathway from further exploration as a potential target in NASH patients.

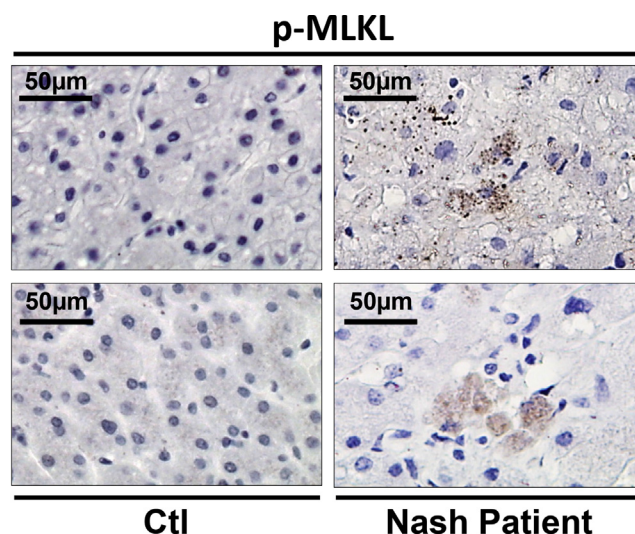
The ideal model to study NASH in mice is a matter of intensive debate. We studied the role of necroptosis in NASH by using the model of methionine-choline-deficient (MCD) diet<sup>3</sup> because this model was used as the experimental basis for the investigation of the role of apoptosis in NASH, leading even to clinical studies in NASH patients.<sup>4</sup> We could demonstrate that mice deficient for *Rip3* show a clear reduction in MCD-diet-induced liver injury, inflammation, and liver fibrosis compared with wild-type mice.<sup>3</sup> We do not know of any other published study challenging this result in the MCD model. In addition, this finding is consistent with a previous study showing similar protective effects of *Rip3*-deletion in a mouse model of alcoholic steatohepatitis.<sup>5</sup> Together, these findings suggest that necroptosis might play an important role in the processes linking NASH with fibrosis.

The authors mentioned inconsistent findings in our study and another study on the role of caspase-8 in the MCD model.<sup>6</sup> Caspase-8 represents an important upstream caspase, initiating the apoptosis process downstream of death receptors such as FAS or tumor necrosis factor (TNF).<sup>7</sup> A newly discovered function of caspase-8 is the suppression of RIP3-dependent necroptosis through cleavage of RIP3 and CYLD.<sup>1,4,8</sup> Consequently, efficient deletion of caspase-8 sensitizes cells to necroptosis through up-regulation of RIP3 expression. This was shown in many different experimental settings and was the case in our studies in which we used a well-established caspase-8 floxed line<sup>9</sup> and the *alp*-cre line mediating robust and efficient deletion of floxed genes in parenchymal livers.<sup>3</sup> Efficient caspase-8-deletion triggered spontaneous RIP3 overexpression (as seen previously in many other organs such as gut or skin<sup>10</sup>), mild liver injury, and

inflammation without any stimulation.<sup>11</sup> Upon MCD diet feeding, caspase-8-deficient mice developed massive RIP3 elevation in hepatocytes and consequently strong liver injury and fibrosis,<sup>3</sup> providing evidence that necroptosis is the main cell death pathway in MCD-diet-induced NASH.

The other important study on caspase-8 in the MCD model cited by the authors used the albumin-cre line for deletion of *Caspase-8*,<sup>6</sup> which did not lead to overexpression of RIP3.<sup>3</sup> Therefore, and for the fact that RIP3 knockout mice were not used, the study by Hatting et al<sup>6</sup> is not suitable to speculate on the role of necroptosis in the MCD model. The authors of the present review article mention that they have preliminary results on a protective role of caspase-8 in a nutritional model of hepatic steatosis (not MCD diet).<sup>2</sup> However, in our work, caspase-8 deletion did not have any effect in a purely nutritional NAFLD model in mice (data not shown). A careful comparison of methodological differences might bring further light into the controversial function of caspase-8 in different NASH models.

Finally, in our study, we had first shown stainings for RIP3 in liver samples of NASH patients, exhibiting a typical granular staining pattern in hepatocytes surrounding areas of high lipid storage.<sup>3</sup> Of note, Western blot analysis and stainings for RIP3 showed a much clearer result than experiments assessing the levels of the cleaved form of the executioner caspase-3 in human NASH livers,<sup>3</sup> suggesting that necroptosis might be at least equally abundant in human NASH as apoptosis. It should be noted that RIP3



**Figure 1. Necroptosis is activated in human nonalcoholic steatohepatitis (NASH) samples.** Representative immunohistochemical staining for phospho-MLKL on livers from different controls (Ctl) and NASH patients. Note the specific granular cytoplasmic staining. See Gautheron et al<sup>3</sup> for the specifics of the NASH cohort as well as the technical and ethical aspects.

overexpression cannot be taken as equal with execution of necroptosis. However, we can now demonstrate that the phosphorylated form of the protein mixed-lineage-like kinase (p-MLKL), which is considered to be the executional kinase of necroptosis,<sup>12</sup> can be detected in human NASH livers (Figure 1) and that it strikingly resembles the granular staining patterns of RIP3 in NASH patients.<sup>3</sup>

Independently of experimental questions in mouse models, we believe that the activation of a known cell-death pathway in hepatocytes in human NASH patients justifies further exploration as a pharmacologic target, potentially as a complementary target to mediators of apoptosis.

JÉRÉMIE GAUTHERON, PhD

MIHAEL VUCUR, PhD

TOM LUEDDE, MD, PhD

Department of Gastroenterology

Digestive Diseases and Intensive Care Medicine

Department of Medicine III

University Hospital RWTH Aachen

Aachen, Germany

## References

- Moriwaki K, et al. *Genes Dev* 2013;27:1640–1649.
- Hirsova P, et al. *Cell Mol Gastroenterol Hepatol* 2015; 1:17–27.
- Gautheron J, et al. *EMBO Mol Med* 2014;6:1062–1074.
- Csak T, et al. *Hepatology* 2011;53:1917–1931.
- Roychowdhury S, et al. *Hepatology* 2013;57:1773–1783.
- Hatting M, et al. *Hepatology* 2013;57:2189–2201.
- Shalini S, et al. *Cell Death Differ* 2014 2014, Published online. <http://dx.doi.org/10.1038/cdd.2014.216>.
- O'Donnell MA, et al. *Nat Cell Biol* 2011;13:1437–1442.
- Salmena L, et al. *Genes Dev* 2003;17:883–895.
- Weinlich R, et al. *Cell Rep* 2013;5:340–348.
- Vucur M, et al. *Cell Rep* 2013;4:776–790.
- Sun L, et al. *Cell* 2012;148:213–227.

## Conflicts of interest

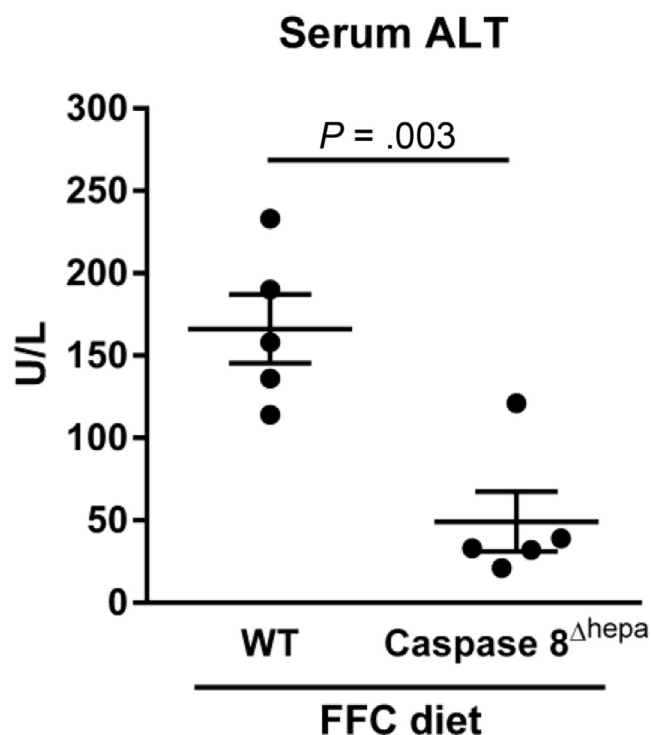
The authors disclose no conflicts.

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**Reply:** We appreciate the interest and thoughtful comments of Dr Luedde and colleagues on our review, “Death Receptor-Mediated Cell Death and Proinflammatory Signaling in Nonalcoholic Steatohepatitis.”<sup>1</sup> As they have made several seminal insights regarding liver pathobiology, we are flattered that our perspectives on nonalcoholic steatohepatitis (NASH), particularly on necroptosis in NASH, triggered their interest. We are happy to continue a friendly scientific dialogue.

They noted, the ideal model of NASH is a matter of intense discussion. We highlight that animals on methionine-choline-deficient (MCD) diet lose weight and



**Figure 1.** NASH-induced liver injury is decreased in hepatocyte-specific caspase 8 knockout mice (caspase 8<sup>Δhepa</sup>). Wild-type mice and mice carrying a hepatocyte-specific deletion of caspase 8 were fed the FFC diet (a NASH-inducing diet high in saturated fats, fructose, and cholesterol) for 3 months, and serum alanine transaminase activity was measured. *P* value was calculated using unpaired two-tailed *t* test.

lack insulin resistance, which is in direct contrast to humans with NASH. From our perspective, the MCD diet model is not informative regarding the mechanisms of steatohepatitis occurring in the context of the metabolic syndrome, and the use of this model should be discouraged.

It is well established that cell death by necroptosis requires that caspase 8 activity be inhibited or disrupted. This requirement may challenge the biological relevance of necroptosis in human liver disease. Currently, only a few conditions are known that lead to disrupted caspase 8 function in humans.<sup>2</sup> For instance, a caspase 8 deficiency state (CEDS), caused by a loss-of-function mutation in the caspase 8 gene, is a very rare genetic disorder of the immune system with no liver phenotype.<sup>3,4</sup> Several viruses and intracellular bacteria may express proteins interfering with caspase 8 activation and can sensitize cells to necroptosis.<sup>5</sup> However, this is not a feature of common hepatotropic viruses such as hepatitis C or B viruses. To date, loss of caspase 8 in steatohepatitis has not been reported. Finally, in preclinical studies of NASH, pharmacologic inhibition of caspases have been associated with beneficial effects, including reduced hepatocyte cell death.<sup>6–8</sup>

As Dr Luedde and colleagues point out, caspase 8 deletion in intestinal or skin epithelium causes receptor-interacting serine/threonine-protein kinase 3 (RIP3)-dependent injury in mice. Similarly, necroptosis may occur