

### Is it appropriate to study blood ghrelin and obestatin in non-alcoholic fatty liver disease (NAFLD) without using protease inhibitors?

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#### To the Editor

I have read the article about the changes in ghrelin and obestatin levels authored by Dr. Gutierrez-Grobe, *et al.*,<sup>1</sup> and published in Annals of Hepatology with great interest. Ghrelin and obestatin are peptide hormones which are easily broken down by proteases.<sup>2,3</sup> These structures which contain less than 50 amino acids are called peptide hormones, while those with more than 50 amino acids are referred to as proteins. Hormones with protein structure are more resistant against proteases. In 2004 Hosada, *et al.* reported the pre-analytical rules that should be followed when studying ghrelin.<sup>2</sup> In accordance with the suggestion of Hosada, *et al.*, a protease inhibitor should be used to protect the ghrelin peptide against proteases.<sup>2</sup> In another study about the optimal stabilization of ghrelin by Blatnik and Soderstrom, the authors stated that protease inhibitors should be used to protect ghrelin against proteases.<sup>3</sup> Furthermore, companies manufacturing kits (Phoenix<sup>4</sup>, bioquote<sup>5</sup>, etc.) all recommend the use of protease inhibitors while studying hormones in peptide structure to protect peptides against proteases. Gutierrez-Grobe, *et al.* studied ghrelin and obestatin changes in patients with non-alcoholic fatty liver disease (NAFLD) and compared those with the levels in control subjects. Total ghrelin levels in NAFLD patients were reported to drop significantly, while obestatin levels also fell, but without a statistically significant difference. Since the au-

thors did not use protease inhibitors while collecting the samples in their study, the decrease in ghrelin and obestatin levels in the patients with NAFLD may be due to the fact that the increased proteases associated with NAFLD has led to the breakdown of these peptides, which were found lower relative to the controls. In NAFLD, the amounts of gamma glutamyl transpeptidase and caspase enzymes increase and these are proteases.<sup>6</sup> These elevated proteases may have broken down the peptides studied and lowered their amounts. A closer examination of the obestatin values that are reported in the study confirms our arguments, as the standard deviation of the obestatin values reported are higher than mean obestatin values. This demonstrates that the amount of protease produced by the patients varies depending on the severity of NAFLD. The protease amounts that vary depending on the severity of the disease affect the rate by which peptides are broken down and lead to higher standard deviations. If the authors had used protease inhibitors in their study, the standard deviations would not have been so high.

The researchers investigated total ghrelin in their study. However, there are two major types of ghrelin in biological fluids. Acylated ghrelin whose 8-C fatty acid is bound to serine amino acid at the N terminal is called desacylated ghrelin when its fatty acid is split. Acylated ghrelin which affects growth hormone secretion and appetite also has antioxidant and anti-inflammatory effects.<sup>7</sup> In other words, the physiological and biochemical effects of acylated and desacylated ghrelin are different. Patients with NAFLD have inflammation besides other clinical signs. Therefore, we believe that measurement of these two forms of ghrelin instead of total ghrelin would be more effective in shedding light on the etiopathology of NAFLD.

There are approximately 700 protease encoded in human genome, according to MEROPS data re-

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cords.<sup>8</sup> Thus, as Gutierrez-Grobe, *et al.*, did not use protease inhibitors in their study,<sup>1</sup> I think that their results should be carefully interpreted in order to decide whether or not peptides really play a role in the aetiology of NAFLD and I also think that it will be appropriate to use protease inhibitors in further studies where peptide concentrations are measured.

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Dear Sir,

We appreciate your interesting comment to our article.<sup>1</sup> Your considerations are pertinent, because it is important to establish a proper protocol for the measurement of this and other proteins. Regarding this issue we follow the instructions from manufacturer (Millipore, MO, USA). The protocol provided indicate clearly the use of AEBSF [4-(2-Aminoethyl)-benzenesulfonyl fluoreide]. This protease inhibitor impedes degradation of the protein,<sup>2</sup> and was used in all our experiments.<sup>1</sup>

However this recommendation brings some interesting issues in patients with non-alcoholic fatty liver disease, which could have higher gamma-glutamyl transpeptidase levels.<sup>3,4</sup> There is a clear effect of proteases over the degradation of protein in stored samples, mainly in those proteins short half life. However, the statement regarding the effect of gamma-glutamyl transpeptidase and other proteases increased in NAFLD is unclear. The reference provided by Aydin is a similar comment by the same author, without ano-

ther reference. We believe there are more important proteases increased in patients with NAFLD, with significant pathogenic implications.<sup>5</sup> Unfortunately the effect of protease inhibitor in the specific setting of NAFLD has not been assessed properly. Anyway their use is highly recommended by the manufacturer, and must be followed in future similar studies.

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