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Mechanistic biomarkers in acute liver injury: Are we there yet?

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Although the outcome of acute liver failure (ALF) has improved due to developments in the general intensive care techniques, mortality rates without transplantation in patients who fulfil poor prognostic criteria are still in excess of 80%. Besides *N*-acetylcysteine, there are no specific treatment options for ALF that occurs on the background of acetaminophen (APAP) toxicity. Also, strategies to limit progression of acute liver injury in patients who are progressing to ALF remain an unmet need. Further difficulties in the management of patients with ALF are the lack of biomarkers that may indicate progression of liver failure early as decision making regarding listing for transplantation in patients with ALF is challenging. At present, the criteria that are used to list patients for urgent transplantation lack sensitivity [1]. Therefore, the paper by Antoine and colleagues [2] in the present issue of the *Journal* is welcome as it starts to address these two areas of unmet need.

The features of APAP-induced hepatotoxicity are apoptosis, necrosis, and innate immune activation [3]. It is important to have a clear understanding of the cellular mechanisms that underlie APAP toxicity as this will not only provide an opportunity to develop biomarkers; the use of these biomarkers will also allow for risk stratification of patients and in doing so, optimise patient care in those presenting following an APAP overdose. In order to address this issue, the authors measured full length keratin-18 (FL-K18) and High Mobility Group Box-1 (HMGB1) as circulating indicators of necrosis, and caspase-cleaved fragment of keratin-18 (cK18) and hyper-acetylated HMGB1 during (APAP) toxicity as serum indicators of apoptosis and immune cell activation, respectively, in patients with APAP-induced acute liver injury and ALF. Their data suggest that these markers accurately reflect severity and pattern of liver injury during its different phases and are potentially important biomarkers that may provide accurate prognostic information.

Keratin-18 (K18) is an intermediate filament responsible for maintaining the cytoskeletal structure in the liver and other epithelial cells. K18 accounts for about 5% of the liver's total protein content. Hepatocyte apoptotic cell death is associated with the release of caspase-cleaved K18 (cK18). Intact full length K18 is released from cells undergoing necrosis [4]. There are sandwich ELISAs available which are able to determine the different forms of K18. The M30 ELISA measures the cK18 released during apoptosis whilst the M65 ELISA detects a common epitope present in the full-length protein as well as caspase-cleaved fragment and is believed to measure both apoptosis and necrosis [4]. Craig *et al.* recently evaluated the use of cK18 and total K18 to aid prognostication in acute liver injury and following APAP overdose. They found that although the total K18 (but not cK18) levels were significantly higher in the patients with APAP-induced acute liver injury, it failed to predict survival [5]. This conflicts with previous results from Volkman *et al.* who suggest that a higher level of cK18 is associated with spontaneous recovery from ALF [6]. In addition, an interesting study by Bechmann *et al.*, which utilised a modified Model for End-Stage Liver Disease (MELD) score, in which serum bilirubin was substituted with K18/M65, demonstrated that the modified M65-based MELD was significantly better at predicting prognosis in ALF patients compared with the current MELD score or Kings College Criteria (KCC) [7].

HMGB1 is a DNA-binding molecule which targets toll-like receptors and the receptor for advanced glycation end products. The protein has different functions depending on its cellular location. Intracellularly, it is involved in transcription, replication, and DNA repair. Outside of cells, it functions as an "alarmin" that can signal danger and traumatic cell death and distress. It is a trigger for inflammation and a stimulus for tissue reconstruction [8]. However, recent data suggest that HMGB1 on its own is insufficient to trigger sustained pro-inflammatory responses and must act in conjunction with molecules such as lipopolysaccharide (LPS) and interleukins to elicit a strong and sustained pro-inflammatory response [8,9]. HMGB1 is released in a hyperacetylated form from innate immune cells and in a hypoacetylated form by necrotic cells [3]. There is a widely used commercially available ELISA kit which measures HMGB1 concentrations in biological fluids [10]. Antoine *et al.* demonstrated in a mouse model of APAP-induced hepatotoxicity that hypoacetylated HMBG1 levels are significantly elevated in the first 3–10 h following APAP hepatotoxicity and that this protein provides valuable information on the histological time course of cell death following APAP hepatotoxicity [3]. The hypo and hyperacetylated forms were determined by mass spectrometry. Following on from this, Craig *et al.* found that although levels of HMGB1 were significantly higher in patients with acute liver injury, there was no significant difference between patients whose liver injury was APAP

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Editorial

Therapeutic targets indicated by biomarkers

Early

Biomarkers	Therapy
Paracetamol level/adducts	N-acetylcysteine

Intermediate

Biomarkers		Targets
Necrosis	Apoptosis	
HMGB1		HMGB1
FL-K18	cK18	TLR4
		LPS
		Liver support

Amplification

Biomarkers	Targets
Acetylated HMGB1	HMGB1
	TLR4
	LPS
	Anti-inflammatory agents
	Liver support

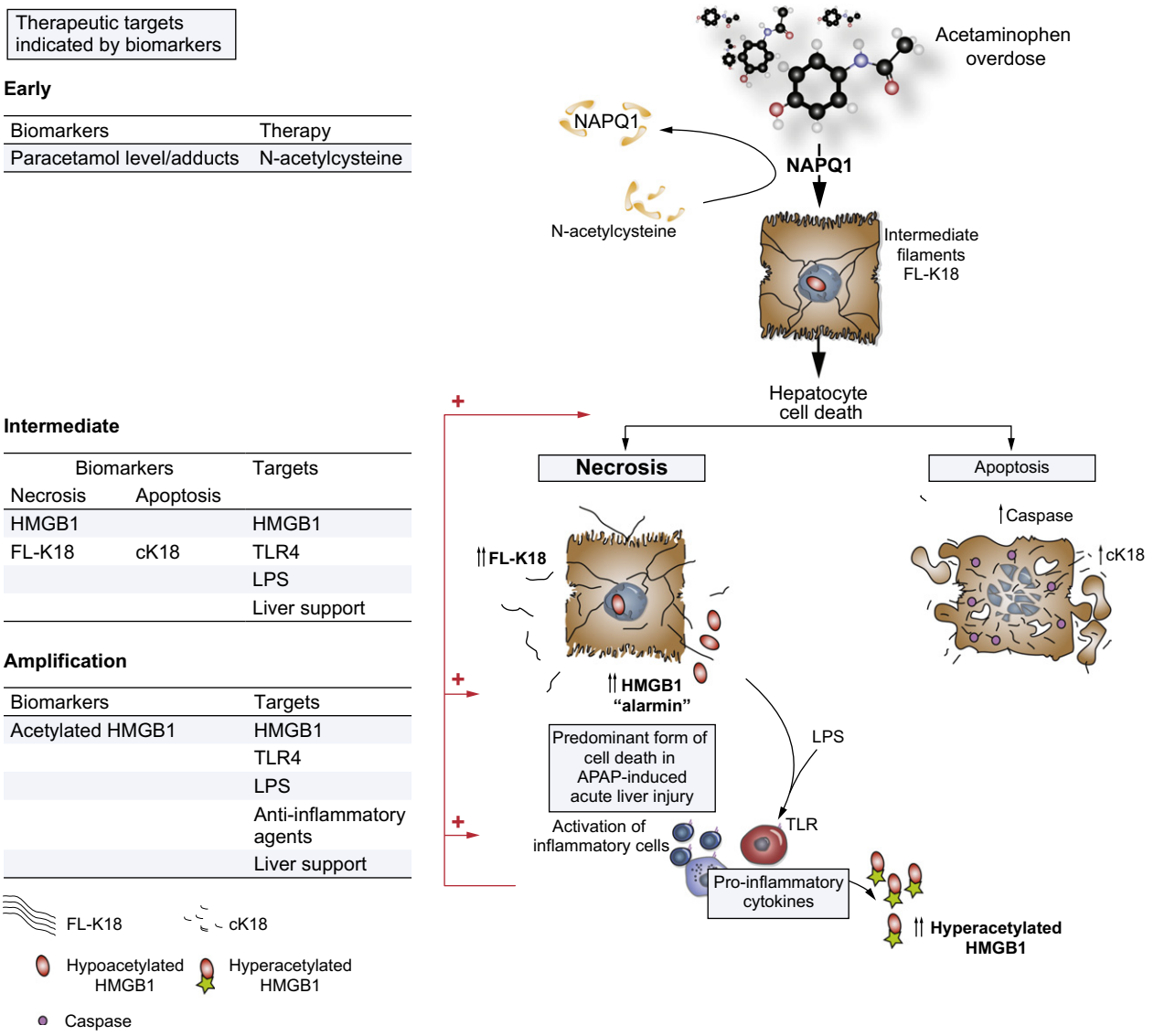

Fig. 1. Potential biomarkers in APAP-induced acute liver injury and therapeutic targets. NAPQI is a toxic metabolite produced in APAP overdose which causes hepatocyte cell death predominantly by necrosis. The necrotic hepatocytes release HMGB1 and full length keratin-18. Cells that die by apoptosis release caspase-cleaved keratin-18. HMGB1 (probably in conjunction with lipopolysaccharide) further amplifies the initial insult by initiating a secondary inflammatory response and increasing the severity of tissue injury.

or non-APAP-induced. In addition, levels of HMGB1 failed to predict survival [5].

The study by Antoine *et al.* [2] adds to the existing data and clarifies some of the apparently discordant results. Eighty-four patients who presented following APAP overdose were categorised into those with normal and abnormal liver function tests (LFTs). The study showed that all the biomarkers (total HMGB1, acetylated HMGB1, cK18, and full-length FL-K18) were significantly elevated in the sera of patients with APAP overdose associated with abnormal LFTs compared to controls. Importantly, there was no significant elevation of these biomarkers in patients with APAP overdose who had normal LFTs suggesting that these biomarkers are sensitive at identifying patients who actually have APAP-induced acute liver injury. As serial analysis of the sera was also carried out, the results showed that necrosis was the predominant form of cell death in the acute phase following APAP overdose as levels of total HMGB1 and FL-K18 were more elevated in the acute phase compared to the markers of apoptosis as has been observed previously [11,12]. Acetylated HMGB1 was elevated in the later stages of APAP overdose in patients who died or required liver transplantation. As the acetylated form of HMGB1 is thought to be derived from activated immune cells, this confirms the findings from other studies which have shown that activation of the innate immune system occurs as a secondary phenomenon following hepatocyte death which may amplify the inflammatory response [13,14]. Samuel *et al.* have previously suggested that an ideal predictive factor for determining prognosis in ALF should be more applicable at predicting death rather than survival. For this reason, the result observed with acetylated HMGB1 is of immense interest [15]. The performance of serial

analysis is commendable as it provides detailed information on the cellular events following APAP-induced liver injury and in so doing, allows for the development of potential targeted therapeutic agents. Fig. 1 illustrates the mechanism by which the various markers of cell death are generated and potential therapeutic targets.

The biomarkers of necrosis (HMGB1 and FL-K18) were both shown to have a strong and significant correlation with prothrombin time, a marker of synthetic liver dysfunction, and ALT activity which is a marker of hepatocellular injury. The mean serum level of all the biomarkers was significantly higher in the patients that fulfilled the KCC poor prognostic criteria compared to patients that did not. The biomarkers of necrosis were more accurate at the prediction of patients with a poor prognosis. Acetylated HMGB1 was "excellent" at predicting which patients met the KCC with an AUC of 0.93 and "good" at predicting which patients were likely to die or require organ transplantation with an AUC of 0.87.

They reported on 'apoptotic index' which is a ratio of cK18 as a proportion of overall K18, which was significantly lower in patients who fulfilled the KCC. It is not clear what this represents and without simultaneous liver biopsy correlation, it remains speculative. Intriguingly, the observation that spontaneous survival following acute liver injury was associated with increased levels of caspase activation is counter-intuitive but has also been made by Volkmann *et al.* [6]. The mechanism through which the increased caspase activation may increase survival is through increased levels of proregenerative cytokines such as IL-6 and TNF-α [6]. The authors did not measure the circulating cytokines and this hypothesis will need to be confirmed in future studies.

The impact of the results generated from this study would be further strengthened by making available the raw data from the M30 (cleaved) and M65 (total) ELISAs, which were used in deriving the results of the full length K18 levels which served as a surrogate marker for necrosis. It would have also been useful to seek histological confirmation on the degree of apoptosis and necrosis from the livers of patients who died or required a liver transplant. Of note, an inter- and intra-assay variability of less than 20% was quoted. For these biomarkers to be useful in clinical practice, one would expect a coefficient of variation which is much less than this.

One of the methods used for HMGB1 determination was the previously mentioned ELISA. Whilst this is a readily available and convenient way to quantify HMGB1 in serum, there is some evidence in the literature that this assay may fail to accurately quantify HMGB1 as various molecules present in the serum may complex with HMGB1 and therefore interfere with its detection by the ELISA technique [16,17]. More studies are therefore needed to investigate methods by which this technique can be improved prior to it being considered for use in the clinical setting. The accurate detection of HMGB1 is particularly important in liver disease. Takano *et al.* have shown that the plasma levels of HMGB1 are elevated in ALF. In addition, their results suggest that HMGB1 neutralising antibodies may have a protective effect against acute liver injury [18]. The growing number of HMGB1 inhibitors in development strengthens the need for a reliable diagnostic tool.

The exciting and novel results from this study pave the way and further highlight the need for additional equally well conducted studies to be carried out so as to ascertain whether biomarkers of cell death in patients with acute liver injury may be of prognostic value and whether they can be used to guide novel approaches to therapy.

Conflict of interest

The author declared that he does not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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