The Role of Mechanistic Biomarkers in Understanding Acetaminophen Hepatotoxicity in Humans

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ABSTRACT
Our understanding of the fundamental molecular mechanisms of APAP hepatotoxicity began in 1973-1974 when investigators at the US National Institutes of Health published seminal studies demonstrating conversion of APAP to a reactive metabolite that depletes glutathione and binds to proteins in the liver in mice after overdose. Since then, additional groundbreaking experiments have demonstrated critical roles for mitochondrial damage, oxidative stress, nuclear DNA fragmentation, and necrotic cell death too. Over the years, some investigators have also attempted to translate these mechanisms to humans using human specimens from APAP overdose patients. This review presents those studies and summarizes what we have learned about APAP hepatotoxicity in humans so far. Overall, the mechanisms of APAP hepatotoxicity in humans strongly resemble those discovered in experimental mouse and cultured hepatocyte models, while emerging biomarkers also suggest similarities in liver repair. The data not only validate the first mechanistic studies of APAP-induced liver injury performed 50 years ago, but demonstrate the human-relevance of numerous studies conducted since then.

SIGNIFICANCE STATEMENT
Human studies using novel translational, mechanistic biomarkers have confirmed that the fundamental mechanisms of APAP hepatotoxicity discovered in rodent models since 1973 are the same in humans. Importantly, these findings have guided the development and understanding of treatments like N-acetyl-l-cysteine and 4-methylpyrazole over the years. Additional research may improve not only our understanding of APAP overdose pathophysiology in humans but also our ability to predict and treat serious liver injury in patients.

NON-STANDARD ABBREVIATIONS
ALF, acute liver failure; ALFSGPI, ALF study group prognostic index; AOPP, advanced oxidation protein products; APAP, acetaminophen; ccK18, caspase-cleaved keratin 18; CLIA, clinical laboratory improvement amendments; ELISA, enzyme-linked immunosorbent assay; LPO, lipid peroxidation; MELD, model for end-stage liver disease; MPT, mitochondrial permeability transition; mtDNA, mitochondrial DNA; NAPQI, N-acetyl-p-benzoquinone imine
INTRODUCTION

Acetaminophen (APAP) is one of the most widely used drugs today (Kaufman et al., 2002; Vernacchio et al., 2009). However, APAP overdose causes severe liver injury, sometimes leading to acute liver failure (ALF) and even death. The phenomenon of APAP hepatotoxicity was first reported in patients in 1966. By the 1990s, it became the dominant single cause of ALF in at least the United Kingdom (O’Grady et al., 1989), the United States (Schiodt et al., 1999), and Denmark (Larsen et al., 1995). It remains one of the most significant causes in many countries to this day (Stravitz and Lee, 2019; Warrillow et al., 2019; Jindal and Sarin, 2022; Lenz et al., 2023).

The mechanisms of APAP hepatotoxicity have been investigated in detail since the 1970s (McGill and Hinson, 2020). These efforts have revealed that APAP is converted to the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) (McGill and Hinson, 2020). This metabolite reacts with both glutathione and proteins (Jollow et al., 1973; Mitchell, Jollow, Potter, Davis, et al., 1973; Mitchell, Jollow, Potter, Gillette, et al., 1973; Potter et al., 1973), and is associated with increased generation of reactive oxygen species (ROS) in mitochondria (Jaeschke, 1990; Knight et al., 2001). The depletion of available glutathione prevents the efficient detoxification of these excess ROS, leading to cell damage. The surplus ROS also trigger activation of various kinases (Nakagawa et al., 2008; Sharma et al., 2012; Ramachandran et al., 2013), which ultimately converge on the c-Jun N-terminal kinases 1/2 (JNK) (Gunawan et al., 2006; Nakagawa et al., 2008). The phosphorylated, active JNK then relocates to mitochondria (Hanawa et al., 2008), leading to a reduction in mitochondrial respiration through the involvement of protein tyrosine phosphatase non-receptor type 6 (PTPN6), also known as SHP1, and Src (Win et al., 2016). Ultimately, the mitochondria undergo depolarization (Kon et al., 2004; Reid et al., 2005), swelling (Petersen and Vilstrup, 1979; Placke et al., 1987), and rupture (Placke et al., 1987), and release endonucleases (Bajt et al., 2006). These endonucleases translocate to the nucleus and cleave nuclear DNA (Bajt et al., 2006, 2011), resulting primarily in oncotic necrosis (Gujral et al., 2002; McGill et al., 2011; McGill, Sharpe, et al., 2012). Certain details of this process remain unclear or are debatable,
like the mechanism by which protein alkylation causes the initial increase in ROS, how these key events cause mitochondria to depolarize, and details of how the liver regenerates after the injury, but our understanding of the pathophysiology of APAP hepatotoxicity overall is relatively clear.

The mechanisms described above were elucidated through decades of research in rodent models, both in vivo and in cell culture. Flashes of insight into the mechanisms of APAP hepatotoxicity in humans have appeared sporadically in the literature throughout the history of APAP overdose as a field of study, but only recently have attempts been made to explore the finer details in earnest through observational studies in patients. Here, I review advances in our understanding of APAP-induced liver injury in humans made using circulating biomarkers since the seminal first mechanistic studies in rodents in 1973, 50 years ago (Jollow et al., 1973; Mitchell, Jollow, Potter, Davis, et al., 1973; Mitchell, Jollow, Potter, Gillette, et al., 1973; Potter et al., 1973, 1974). The clinical utility of some of these biomarkers will also be discussed.

BRIEF HISTORICAL PERSPECTIVE: APAP HEPATOTOXICITY MECHANISMS IN NON-ClinICAL MODELS

The mechanisms of APAP hepatotoxicity have been reviewed in detail elsewhere (McGill and Jaeschke, 2013; Iorga et al., 2017; McGill and Hinson, 2020; Ramachandran and Jaeschke, 2020; Jaeschke and Ramachandran, 2023). This section provides a brief overview. For more detailed information, the reader is directed to the reviews cited above, including one appearing in this issue of the journal (Jaeschke and Ramachandran, 2023).

*Glutathione depletion and protein binding.* Seminal studies by David Jollow, Jerry Mitchell, Bernard Brodie, Jim Gillette and others at the US National Institutes of Health, published in 1973-74, laid the foundation for both the treatment of APAP overdose with N-acetyl-l-cysteine – and more recently 4-methylpyrazole – and the mechanistic exploration of APAP toxicity (Jollow et al., 1973; Mitchell, Jollow,
Potter, Davis, *et al.*, 1973; Mitchell, Jollow, Potter, Gillette, *et al.*, 1973; Potter *et al.*, 1973, 1974). These authors demonstrated in rodents that acetaminophen is metabolized by cytochromes P450 enzymes to a reactive metabolite which depletes glutathione and binds to proteins. Interventions that blocked P450 activity blocked toxicity, while interventions that increased P450 activity increased toxicity. The latter data demonstrated the clear role of protein alkylation in the initiation of APAP hepatotoxicity.

*Mitochondrial damage and oxidative stress.* Mitochondrial dysfunction is also a central feature of APAP hepatotoxicity in rodents. The authors of the 1973-74 papers noted mitochondrial protein alkylation (Jollow *et al.*, 1973), but did not comment on its importance. Later histopathological examination of liver sections from an APAP overdose patient revealed decreased mitochondrial number and altered mitochondrial morphology (swelling and loss of cristae) (Petersen and Vilstrup, 1979). It was also reported in 1979 that mM concentrations of APAP could reduce mitochondrial respiration in isolated renal tubules and tubular cells (Porter and Dawson, 1979), which are known to express P450s. Full recognition of the importance of mitochondria came in the 1980s. In 1988, Meyers et al. reported that APAP overdose inhibits mitochondrial respiration in the mouse liver in a P450-dependent manner (Meyers *et al.*, 1988). This fundamental observation was quickly reproduced by others (Andersson *et al.*, 1990; Burcham and Harman, 1990). Shortly thereafter, Jaeschke reported preferential glutathione oxidation within hepatic mitochondria in APAP overdosed mice (Jaeschke, 1990), indicating a compartmentalized mitochondrial oxidative stress consistent with the absence of increased GSSG in bile after APAP treatment that he had previously observed (Smith and Jaeschke, 1989; Jaeschke, 1990). Later experiments indicated that the major mitochondrial ROS involved are superoxide (O$_2^-$) and peroxynitrite (ONOO$^-$) (Knight *et al.*, 2001). It was also later discovered that these effects on mitochondria likely depend upon mitochondrial protein alkylation (McGill, Williams, *et al.*, 2012; Xie, McGill, Du, *et al.*, 2015) and eventually cause the mitochondrial membrane permeability transition (MPT) (Kon *et al.*, 2004; Reid *et al.*, 2005). Recent studies have also demonstrated that genetic and pharmacological interventions that mitigate the MPT or mitochondrial oxidative stress also reduce the liver injury (Ramachandran,
Lebofsky, Baines, et al., 2011; Lee et al., 2015; Du et al., 2017; Nguyen et al., 2021; Adelusi et al., 2022), while those that worsen the oxidative stress also increase injury (Ramachandran, Lebofsky, Weinman, et al., 2011; Yiew et al., 2023).

The role of JNK. It has been appreciated for some time that JNK has a role in various cell death signaling pathways. A possible role for JNK in APAP toxicity, specifically, has been considered since the 1990s. Schwenger et al. looked for JNK activation in COS-7, HT-29, and FS-4 cells treated with 800 µM APAP but found none (Schwenger et al., 1999). This is not surprising given the fact that these cell lines are not known to express P450s and because of the APAP concentration they used. Two years later, however, Bae et al. published a study in which they treated C6 cells with 1 to 10 mM APAP and observed evidence of JNK1 activation preceding or co-incident with cytotoxicity (Bae et al., 2001). They also found that genetic inhibition of JNK activity partially decreased the cell injury (Bae et al., 2001). However, the relevance of those data to P450-expressing hepatocytes was, again, questionable. Around the same time, Kevin Park’s group in Liverpool proposed that JNK might be protective in APAP hepatotoxicity based on indirect evidence (Henderson et al., 2000; Elsby et al., 2003). Finally, the role of JNK in APAP toxicity was directly tested in hepatocytes. It was discovered that JNK is phosphorylated and activated after APAP overdose in mice and mouse hepatocytes and that various approaches to reduce JNK activation were protective (Gunawan et al., 2006; Henderson et al., 2007; Latchoumycandane et al., 2007). We now know that the role of JNK is initiated by oxidative stress through upstream kinases like apoptosis signal-regulating kinase 1 (ASK1) (Nakagawa et al., 2008) and mediated by other, downstream proteins; namely, Sab and Src (Win et al., 2016). It appears that the activated JNK translocates to mitochondria and worsens mitochondrial oxidative stress by inhibiting Src and reducing mitochondrial respiration, likely leading to accumulation of reduced electron carriers which donate electrons to O₂ to form O₂⁻, enhancing oxidative damage (Win et al., 2011, 2016, 2019; McGill and Hinson, 2020).

DNA degradation. Nuclear DNA fragmentation was first described in APAP-induced liver injury in mice in 1990 (Ray et al., 1990). Earlier studies had noted increased unscheduled DNA synthesis in
hepatocytes after APAP treatment (Dybing et al., 1984), indicating DNA repair, but had not directly assessed the type of DNA damage in mouse livers. Studies in the 1990s revealed that the damage includes double-strand breaks. Initially, this fragmentation was thought to be due to a Ca\(^{2+}\)-dependent endonuclease, as mentioned by Ray et al. (Ray et al., 1990). However, we now understand that the mitochondrial damage described above leads to release of the enzymes Endonuclease G and apoptosis-inducing factor (AIF) from the mitochondrial intermembrane space and these enzymes are largely responsible for the nuclear DNA cleavage after translocation to the nucleus (Bajt et al., 2006, 2011). Other endonucleases may also contribute, such as DNase1 (Napirei et al., 2006; Koyama et al., 2016).

*Mode of cell death.* The observation that nuclear DNA fragmentation occurs in the murine liver after APAP overdose led to the idea that APAP causes hepatocyte death via apoptosis (Ray et al., 1990). At the time, the concept of apoptosis was relatively new (Kerr et al., 1972) and DNA fragmentation had recently been reported as a characteristic apoptotic feature (Wyllie, 1980). However, later work found little to no evidence of hepatic caspase 3 activation after APAP overdose in mice, indicating that apoptosis does not play a role in APAP hepatotoxicity (Lawson et al., 1999). Indeed, detailed morphological analysis of liver sections revealed that <1% of hepatocytes appeared apoptotic in APAP-induced liver injury in rodents compared to about 24% after combined galactosamine/endotoxin treatment as a positive control for apoptosis (Gujral et al., 2002). Furthermore, caspase inhibitors failed to protect against APAP in mice (Jaeschke et al., 2006; Williams et al., 2010). Together, these data and the fact that most hepatocytes appear necrotic after APAP overdose (Gujral et al., 2002) demonstrated that the primary mode of cell death in APAP hepatotoxicity in rodents is necrosis. Furthermore, a few recent studies have demonstrated that programmed necrosis, specifically, plays an important role in APAP liver injury (Ramachandran et al., 2013; Zhang et al., 2014; Jaeschke and Ramachandran, 2020), though some of these data have been contested (Dara et al., 2015).
KEY ADVANCES: TRANSLATIONAL MECHANISTIC STUDIES IN HUMANS USING BIOMARKERS

Over the years, attempts have been made to translate the mechanisms of APAP hepatotoxicity described above to humans using human specimens. The following section summarizes those efforts, broadly following the progression of mechanisms in the previous section. The studies are also summarized in Table 1, with graphical representation in Figures 1 and 2.

Evidence of glutathione depletion and protein alkylation in humans after APAP overdose from blood and urine studies. Jagenburg and Toczko first detected APAP-cysteine derivatives in urine from human subjects administered either phenacetin, an APAP precursor, or APAP itself in 1964 (Jagenburg and Toczko, 1964). This was prior to the mechanistic studies of 1973 and the authors did not comment on the possibility of reactive metabolite formation. Nevertheless, the data support the idea that humans also form a reactive metabolite of APAP that binds to glutathione. Davis et al. (Davis et al., 1975) later examined bromosulphthalein (BSP) clearance in volunteers administered 2 g of APAP and in APAP overdose patients and found that it was prolonged. BSP is a dye that is eliminated from the body primarily via glutathionylation in the liver. Prolonged clearance may thus indicate either loss of glutathione or liver dysfunction. The elimination rates of BSP from plasma were reduced 30-90% in APAP overdose patients compared to healthy volunteers. Importantly, the latter effect was likely not entirely due to liver damage because the authors also found that BSP elimination rates were approximately 25% lower in healthy volunteers after taking non-toxic APAP doses compared to before dosing (Davis et al., 1975). BSP retention and the percentage of plasma BSP in the free, unconjugated form also increased in the APAP groups (Davis et al., 1975). These data again provided evidence that glutathione is depleted in APAP overdose in humans. Later, Lauterburg and Mitchell administered radiolabeled cysteine to human volunteers followed by various doses of APAP, isolated the glutathione-derived APAP mercapturic acid metabolite, and measured radioactivity (Lauterburg and Mitchell, 1987). They not only detected the radiolabel in the mercapturic acid metabolite, providing direct evidence of NAPQI binding to GSH, but
also observed that doses ≥600 mg increased glutathione turnover compared to lower doses, thus demonstrating increased GSH demand (Lauterburg and Mitchell, 1987). Altogether, these data strongly indicate that glutathione binding occurs in humans after APAP exposure, like mice (Table 1).

The first report of APAP-protein binding in humans was published in 1990. Hinson et al. had previously developed an immunoassay for protein-bound APAP-cysteine in mice (Roberts et al., 1987). In a seminal study, they used their immunoassay to detect and quantify APAP-protein adducts in serum from 30 APAP overdose patients (Hinson et al., 1990). They also reported that patients treated with NAC ≥8 hours after overdose, and who had the greatest risk of hepatotoxicity based on the Rumack-Matthew nomogram, had the highest adduct values. Similar results have been reported in numerous studies since then (Davern et al., 2006; James et al., 2006, 2009; Cook et al., 2015; Xie, McGill, Cook, et al., 2015; Curry et al., 2019). It is therefore clear that glutathione loss and APAP-protein binding both occur in humans (Table 1).

Interestingly, APAP-protein adducts are detectable even in serum from subjects taking therapeutic APAP doses, raising the question of how adducts are released into the circulation in the absence of cell death and loss of plasma membrane integrity. Recent data have shed light on the possible mechanisms. Three hypotheses have been considered: 1) Adduction of proteins followed by secretion of the proteins into blood; 2) diffusion of NAPQI from hepatocytes into the blood, where the NAPQI reacts with proteins in situ; and 3) transport of adducts into blood through extracellular vesicles. To begin exploring these possibilities, we treated primary mouse hepatocytes with APAP in the absence of extracellular protein and demonstrated that APAP-protein adducts were still detectable in the culture medium (McGill et al., 2013). Importantly, APAP did not cause cytotoxicity in these conditions and the appearance of adducts in the medium coincided with the appearance of secreted proteins, consistent with the first hypothesis. In addition, in the presence of added extracellular protein, APAP was cytotoxic and this was accompanied by greater values for APAP-protein adducts in the culture medium, consistent with release of additional adducts due to necrosis. Although the second hypothesis could also explain the
increased adducts in medium in the cultures with added extracellular protein, that explanation is less parsimonious and there is no direct evidence to support it. Thus, the idea that significant NAPQI diffusion occurs, as in the second hypothesis, is not supported by currently available data. It is also inconsistent with the known rapid reaction rate of NAPQI with cellular thiols such as glutathione (Coles et al., 1988), which would be expected to quickly neutralize NAPQI before it could travel. Finally, Duan et al. addressed the third hypothesis. They isolated extracellular vesicles from culture medium of primary mouse hepatocytes and HepaRG cells exposed to APAP, from plasma from mice overdosed with APAP, and from plasma from APAP overdose patients, and measured APAP-protein adducts (Duan et al., 2019). Adducts were undetectable in EVs from all three models (Duan et al., 2019), effectively ruling out vesicular release as well as any formation of NAPQI or adducts within EVs themselves as others have suggested may occur (Kumar et al., 2017). While the authors used only large doses and concentrations of APAP that do not reflect therapeutic APAP use, they did observe increased EV production in mice at 2 h after APAP treatment – before the onset of liver injury – and still could not detect APAP-protein adducts in those EVs (Duan et al., 2019). On the other hand, adducts were readily detectable in plasma. Thus, overall, secretion of adducted proteins formed within hepatocytes – the first hypothesis – seems to be the most likely explanation for the appearance of adducts in circulation prior to or in the absence of liver injury, though it should be noted that necrosis can lead to even greater adduct release (McGill et al., 2013; Thomas et al., 2016; Curry et al., 2019).

Biomarkers of mitotoxicity and increased ROS after APAP overdose in humans. It is difficult to obtain liver tissue from APAP overdose patients. Thus, we decided on a comparative approach to investigate the molecular pathophysiology of APAP hepatotoxicity in humans using blood. To determine if mitochondrial damage occurs in humans in the liver after APAP overdose as it does in mice, we measured mitochondrial macromolecules in plasma from APAP overdose patients and performed parallel experiments in mice to demonstrate specificity of those markers for mitochondrial damage (McGill, Sharpe, et al., 2012). Comparison of plasma glutamate dehydrogenase (GLDH) and mitochondrial DNA
(mtDNA) between mice treated with hepatotoxic doses of APAP and furosemide – mitochondria not being a target of the latter (Wong et al., 2000) – indicated that these macromolecules are only elevated, or are elevated more, when the mechanism of liver injury involves mitochondrial damage. Recent work has reproduced this fundamental observation (Church et al., 2020). Although the authors of the latter study presented their results as a challenge to our work because GLDH was technically elevated in the furosemide model in their hands, the elevation they reported after furosemide treatment was still far lower than the elevation they observed in APAP-treated mice (Church et al., 2020; McGill and Jaeschke, 2021). This preferential release of mitochondrial content after APAP is likely because the damaged mitochondria spill their contents into the cytosol (Placke et al., 1987), facilitating greater release into circulation upon cell necrosis (Figure 1). Critically, GLDH and mtDNA are also dramatically elevated in plasma from APAP overdose patients with liver injury (McGill, Sharpe, et al., 2012). Together, these data indicate that mitochondrial damage occurs in humans after APAP overdose, as it does in mice (Table 1). We also observed nuclear DNA fragmentation in those patients (McGill, Sharpe, et al., 2012), and DNA fragmentation in APAP hepatotoxicity is known to involve endonucleases released from damaged mitochondria (Bajt et al., 2006, 2011). Moreover, using the same approach we used with GLDH and mtDNA, we demonstrated that long-chain acylcarnitines are biomarkers of mitochondrial damage (McGill, Li, et al., 2014) and others have reported acylcarnitine elevations in APAP overdose patients under certain circumstances (Bhattacharyya et al., 2014).

Other groups have also reported increased mitochondrial damage biomarkers in APAP overdose patients. The biomarker carbamoyl phosphate synthetase 1 (CPS1) is an example. In a 2006 study, it was revealed that circulating CPS1 levels increased at both 8 and 24 hours following surgery in a sepsis-induced liver damage model (Crouser et al., 2006). Significantly, this CPS1 surge coincided with the onset of mitochondrial depletion (possibly due to mitochondrial destruction) in the liver and with alterations in mitochondrial morphology indicative of damage. In contrast, the release of ALT, a liver enzyme, occurred at a considerably later stage. The authors of the study interpreted these findings to mean
that CPS1 entered the bloodstream due to mitochondrial damage, rather than as a mere secondary effect of necrosis (Crouser et al., 2006). Consistent with the latter, it was recently observed that CPS1 is selectively released by isolated mitochondria and mouse hepatocytes under hypo-osmotic conditions and that this is accompanied by large changes in mitochondrial morphology (Li et al., 2023). In hepatocytes in particular, the authors saw swelling of mitochondria accompanied by the preferential release of CPS1 into the cytosol and then into the culture medium (Li et al., 2023). Similarly, they observed CPS1 release into bile in a mouse model of hyponatremia, though it did not appear in serum, possibly because “the extent of liver injury after water administration was relatively mild.” Altogether, these data are consistent with the idea that CPS1 release into circulation indicates mitotoxicity. Importantly, Weerasinghe et al. measured CPS1 in blood from APAP overdose patients and found that it was elevated (Weerasinghe et al., 2014), again supporting the idea that mitochondrial damage occurs in humans during APAP hepatotoxicity (Table 1). Interestingly, GLDH, mtDNA, nuclear DNA fragments, and CPS1 are also elevated in non-survivors of APAP-induced ALF compared to survivors (McGill, Staggs, et al., 2014; Kwan et al., 2023). These data may indicate that mitochondrial damage not only occurs in APAP overdose patients, but is a primary driver of the liver injury as it is in mice (McGill and Jaeschke, 2014).

Another potential biomarker of mitochondrial damage after APAP overdose that has not yet been measured in humans is cytochrome c. Miller et al. observed translocation of cytochrome c from mitochondria to the cytosol after APAP overdose or galactosamine/endotoxin treatment in rats, based on changes in immunohistochemistry, suggesting release due to mitochondrial dysfunction (Miller et al., 2008). They were then able to detect elevated cytochrome c in serum from both the APAP and endotoxin-treated animals and in urine from the endotoxin animals (Miller et al., 2008). Thus, cytochrome c may be another useful marker of mitochondrial damage in humans, though that remains to be tested.

Surprisingly, much less work has been done to explore oxidative stress in human APAP hepatotoxicity, despite the availability of numerous convenient endpoints to study it, including protein carbonyls, 8-OHdG, F₂ isoprostanes, advanced oxidation protein products (AOPPs), ischemia-modified
albumin, nitrotyrosine, dityrosine dimers, and so on (Murphy et al., 2022). Nevertheless, there is some evidence for oxidative stress in humans after APAP overdose. Elevated F$_2$-isoprostanes have been reported in blood or urine samples from patients with APAP-induced liver injury in at least two studies (Morrow et al., 1993; Awad et al., 1996; Delanty et al., 1996; Morrow, 2000), consistent with oxidative stress occurring in humans as it does in rodents. This is interesting, as F$_2$-isoprostanes are usually considered evidence of lipid peroxidation (LPO) but LPO does not have a significant role in the mechanisms of APAP hepatotoxicity in rodents under normal conditions (Knight et al., 2003; Adelusi et al., 2022). However, it is possible that some incidental LPO occurs as an epiphenomenon or a “spillover effect” of the overall oxidative stress, even if it does not drive the liver injury per se. In contrast, in another study, the investigators measured several purported markers of oxidative stress in patients with various drug overdoses, including APAP overdose (Hydzik et al., 2016) and found no evidence of oxidative stress. Except for a decrease in serum catalase of questionable biological relevance, they found no significant differences between the APAP patients and healthy controls for any endpoint, including protein carbonyls, which have been detected in hepatic mitochondria from mice with APAP hepatotoxicity (Bajt et al., 2011). Unfortunately, the paper contains insufficient clinical detail to determine the rigor and significance of the results. For example, there is no information in the manuscript about the incidence or severity of liver injury among the APAP overdose patients, nor about the time frame of NAC treatment which would be expected to have strong antioxidant effects. Interestingly, a study of patients with idiosyncratic drug-induced liver injury from the Drug-Induced Liver injury Network (DILIN) revealed that they had elevated levels of serum AOPPs and ischemia-modified albumin (Xiao et al., 2020). While the DILIN generally excludes APAP overdose patients, elevated plasma AOPPs have also been observed in rodents with APAP hepatotoxicity and the plasma kinetics resembled the kinetics of dityrosine immunostaining in the liver (Sun et al., 2011). Together, these data indicate that AOPPs may be elevated in blood from APAP overdose patients too, though that remains to be investigated with rigorous methods. Overall, data regarding oxidative stress in APAP hepatotoxicity in humans are sparse, but suggest that oxidative stress likely does occur (Table 1). This especially seems true when considered together with
evidence of oxidative stress in APAP-exposed human hepatocytes (McGill et al., 2011), including JNK activation (Xie et al., 2014). However, more research is needed in this area.

**DNA fragments in circulation.** As mentioned above, nuclear DNA fragments have also been measured in circulation from APAP overdose patients with liver injury. Craig et al. first reported the presence of nucleosomes in serum from patients with ALF of multiple etiologies, including APAP overdose, using an ELISA with capture antibodies against histones and a detection antibody against DNA (Craig et al., 2011). These results have been reproduced in multiple studies since then (McGill, Sharpe, et al., 2012; McGill, Staggs, et al., 2014). Recently, Laurent et al. analyzed total circulating cell-free DNA (cfDNA) in APAP overdose patients and found that it too is elevated (Laurent et al., 2020). Consistent with the earlier nucleosome immunoassay data, however, they also analyzed cfDNA in plasma from mice with APAP hepatotoxicity and found that most of the increased cfDNA existed in the form of 170 bp fragments (Laurent et al., 2020) — similar in size to single nucleosomes. Thus, nuclear DNA fragmentation occurs in humans with APAP hepatotoxicity (Table 1). Future studies should focus on verifying that this fragmentation is secondary to mitochondrial in humans, as it is in mice.

**Cell death biomarkers.** A dominant role for necrosis in APAP hepatotoxicity in patients is supported by data for total HMGB1, keratin 18 (K18), caspase-cleaved K18 (ccK18), and caspase 3 activity in circulation. There is evidence that HMGB1 is released during necrosis but localizes to the nucleus and stays within cells during apoptosis (Scaffidi et al., 2002). Importantly, elevated HMGB1 has been reported in multiple studies of APAP overdose patients (Craig et al., 2011; Dear et al., 2018), indicating necrosis of hepatocytes. K18 and ccK18 allow measurement of both necrosis (total K18) and apoptosis (ccK18). Consistent with data regarding cell death mode in mice (Gujral et al., 2002) and in cultured human hepatocytes (McGill et al., 2011), most K18 in serum from APAP overdose patients with liver injury exists in the full-length form. Only about 10-20% exists as ccK18 (Volkmann et al., 2008; Craig et al., 2011). Finally, despite seeing elevated caspase 3 activity in plasma from mice with hepatocyte apoptosis due to galactosamine/endotoxin treatment, we could not detect any such activity in
plasma from APAP overdose patients with liver injury or in mice overdosed with APAP (McGill, Sharpe, et al., 2012). Overall, it appears that necrosis is the major mode of cell death in APAP overdose patients, as it is in mice (Table 1).

**Other potential mechanistic biomarkers.** It was recently proposed that bile acids also mediate APAP hepatotoxicity (Table 1). Accumulation of bile acids within hepatocytes was observed early after APAP overdose in mice and blocking hepatic re-uptake of bile acids reduced the toxicity (Ghallab et al., 2022). Furthermore, it has been known for years that bile acid concentrations are elevated in circulation after APAP overdose in humans (Woolbright et al., 2014; James et al., 2015; Luo et al., 2018; Ghallab et al., 2022). In fact, Woolbright et al. demonstrated that serum glycodeoxycholic acid levels, in particular, are associated with death in patients with APAP-induced ALF (Woolbright et al., 2014). Thus, bile acids may be viewed as mechanistic biomarkers in APAP overdose as well. On the other hand, seemingly conflicting data have demonstrated that blocking enterohepatic cycling of bile acids using cholestyramine actually worsens APAP hepatotoxicity in mice (Bhushan et al., 2013). Thus, additional studies are needed to fully elucidate the role of bile acids in APAP-induced liver injury before firm conclusions can be drawn.

**The emerging mechanistic significance of alanine aminotransferase.** Alanine aminotransferase (ALT) was introduced as a general biomarker of liver injury in the 1950s (De Ritis et al., 1955; Karmen et al., 1955). It became the primary liver injury biomarker within a decade – around the same time that the first reports of APAP overdose were published (McGill and Curry, forthcoming publication). There are two isoforms of ALT: a cytosolic form, ALT1, and a mitochondrial form, ALT2 (McGill, 2016). Both enzymes are important for pyruvate and glucose metabolism. Surprisingly, however, despite their critical functions and their long association with liver injury as biomarkers, few studies have investigated the role of ALT enzymes in liver pathophysiology. Using pharmacological and genetic approaches, we recently demonstrated that ALT2 has an important role in the maintenance and recovery of hepatic glutathione levels, in conjunction with the mitochondrial pyruvate carrier (MPC) complex (Yiew et al., 2023).
deletion or inhibition of either the MPC or ALT2 alone had no effect on APAP hepatotoxicity in mice, knockout of both resulted in lower liver glutathione levels at baseline and prolonged glutathione depletion and greater oxidative stress after APAP overdose, leading to greater liver injury (Yiew et al., 2023). This was likely the result of increased glutaminolysis and diversion of glutamine/glutamate away from glutathione synthesis. Conversely, enhancement of pyruvate flux, either by inhibiting pyruvate dehydrogenase kinase with dichloroacetate or by induction of ALT2 with dexamethasone, protected against APAP hepatotoxicity (Yiew et al., 2023). Other recent data have shown that ALT has a role in gluconeogenesis in diabetes and metabolic liver disease as well (McCommis et al., 2015; Martino et al., 2022). Thus, while ALT may not be a mechanistic biomarker per se, it is interesting that the ALT enzymes play a long-overlooked role in APAP hepatotoxicity, and in liver disease more broadly.

**Inflammation biomarkers.** After APAP overdose, the initial liver damage is followed by a sterile inflammatory response in which damage associated molecular patterns (DAMPs; e.g. nuclear DNA fragments, mtDNA) released from the dying hepatocytes activate pro-inflammatory receptors, like toll-like receptors on macrophages, and stimulate production of cytokines and chemokines which attract and activate still other inflammatory cells (e.g. neutrophils, natural killer T cells, infiltrating monocytes) (Fig. 2). Mixed data indicate that this inflammation may or may not propagate injury at later stages and suggest that it can contribute to liver repair and recovery (Laskin and Pilaro, 1986; Laskin et al., 1986, 1995; Dambach et al., 2002; Ju et al., 2002; Liu et al., 2006; Holt et al., 2008; Imaeda et al., 2009; Williams et al., 2010, 2011, 2014; Hoque et al., 2012; Nguyen et al., 2023). While the exact effects of the cytokines, chemokines, and other inflammation signaling mediators involved remain hazy, it is clear that they do have some role.

The most consistently elevated cytokines in APAP overdose patients appear to be interleukin (IL)-6, IL-8, and monocyte chemoattractant protein 1 (MCP1) (James et al., 2001, 2004, 2005; Williams et al., 2003; Woolbright et al., 2022). Of these, the role of IL-6 in APAP toxicity has been most thoroughly investigated in experimental models. The experiments that have most directly addressed the
role of IL-6 have shown that exogenous IL-6 protects against APAP-induced liver injury (Gao et al., 2019) while IL-6 KO may worsen it (Clemens et al., 2021) and delay liver regeneration (James et al., 2003). Nevertheless, there are conflicting data with respect to IL-6 (Bourdi et al., 2007). Similar confusion exists with regard to the role of MCP-1. While at least one study has shown worse APAP hepatotoxicity in mice deficient in the MCP-1 receptor C-C chemokine receptor 2 (CCR2) (Hogaboam et al., 2000), another found no consistent, significant effect on injury (Dambach et al., 2002). Recent data indicate that such conflicts may be due to differences in study design, such as the use of different doses across studies (Nguyen et al., 2023). Other cytokines, chemokines, and other pro-inflammatory signaling mediators (aside from nuclear DNA fragments, mtDNA, and others discussed above) that have been reported to be increased in circulation of APAP overdose patients include CXCL14 (Umbaugh et al., 2024) and leukocyte cell-derived chemotaxin 2 (Lect2) (Slowik et al., 2019).

Liver regeneration biomarkers. The fundamental mechanisms of liver repair and regeneration after APAP overdose are underexplored (MMM Clemens et al., 2019). Nevertheless, some recent studies have shed light in this area and we can begin to explore mechanistic biomarkers of liver regeneration. For example, we now know that IL-6 increases early after APAP overdose in mice and that IL-6 KO delays regeneration (James et al., 2003). We also know that β-catenin signaling plays a major role in driving the regeneration, as inhibition of the β-catenin reduces regeneration while inhibition of the β-catenin regulator glycogen synthase kinase 3β (GSK3β) enhances it (Apte et al., 2009; Bhushan et al., 2014; Bhushan, Poudel, et al., 2017; MM Clemens et al., 2019; Poudel et al., 2021). In addition, endothelial growth factor receptor (EGFR) also appears to be important (Bhushan, Chavan, et al., 2017). Finally, certain metabolic signals have been proposed to enhance regeneration, including bile acids and phosphatidic acids (Bhushan et al., 2013; Lutkewitte et al., 2018; MM Clemens et al., 2019).

In addition to IL-6 discussed in the previous section, bile acids (Woolbright et al., 2014; Ghallab et al., 2022) and phosphatidic acid (Lutkewitte et al., 2018) are elevated in blood from APAP overdose patients and may therefore serve as biomarkers of liver regeneration. However, more research is needed
to confirm those reports. Aside from these potential mechanistic regeneration biomarkers, several other biomarkers of liver regeneration are available or have been proposed. For example, liver function tests such as serum bilirubin and prothrombin time could be considered regeneration markers because a return to normal values for these tests after injury indicates recovery of liver function. The tumor marker α-fetoprotein (AFP) is elevated in immature, proliferating hepatocytes, indicating liver regeneration in the context of APAP hepatotoxicity, and has been proposed as a biomarker of liver regeneration as well (Schmidt and Dalhoff, 2005). Other potential regeneration markers that have been proposed for use in APAP overdose include serum phosphate (Schmidt and Dalhoff, 2002) and α-NH₂ butyric acid (Rudnick et al., 2009).

CLINICAL UTILITY OF MECHANISTIC BIOMARKERS

Recent studies have shown that several of the mechanistic biomarkers discussed in this paper may have prognostic utility to predict death and therefore improve donor organ allocation in the context of APAP-induced ALF. The mitochondrial damage biomarkers GLDH, mtDNA, and nuclear DNA fragments are associated with death (McGill, Staggs, et al., 2014), indicating that mitochondrial damage is a critical feature of APAP hepatotoxicity in humans. However, their sensitivity and specificity are relatively poor (McGill, Staggs, et al., 2014). CPS1, another of the mitochondrial damage markers, was also associated with death in ALFSG patients. Kwan et al. measured CPS1 in longitudinal serum samples from 103 acute liver failure patients with APAP-induced injury and 167 patients with injury of other etiologies. They found that CPS1 values were higher in APAP overdose patients with poor outcomes (defined as death or liver transplant) after APAP hepatotoxicity, though this difference was not present in non-APAP patients. Furthermore, the addition of CPS1 to the Acute Liver Failure Study Group Prognostic Index (ALFSGPI) very modestly improved positive and negative predictive values for poor outcomes in the APAP group (Kwan et al., 2023).
The inflammation markers Lect2 (Slowik et al., 2019) and CXCL14 are also elevated in non-survivors of APAP-induced ALF (Umbaugh et al., 2024). Lect2 expression in hepatocytes increases during injury and the protein is subsequently released with hepatocyte damage and activates inflammatory cells. Slowik et al. thus measured Lect2 in 42 survivors and 19 non-survivors of ALF, most of whom (82%) had APAP-induced ALF (Slowik et al., 2019). Lect2 concentration was higher overall in the non-survivors. While it was only weakly associated with death on its own (Odds Ratios [OR] for survival of 0.92-0.98), the MELD score did not perform much better in their patients (OR: 0.89). Furthermore, the combination of Lect2 and other markers such as INR, albumin, and the MELD score showed greater promise for prognosis (Slowik et al., 2019). CXCL14 is another chemokine released from hepatocytes. Umbaugh et al. recently measured CXCL14 in plasma or serum from two cohorts of APAP overdose patients: 50 patients enrolled at sites in Arizona and Kansas and 80 enrolled through the ALFSG (Umbaugh et al., 2024). CXCL14 had very high sensitivity (78-100%) and specificity (79-100%) for poor outcomes in the first cohort, with lower but still impressive values (70-80%) in the second. Further analyses using simulated populations yielded similar results. Their data also indicated that CXCL14 has potential to outperform the conventional model for end-stage liver disease (MELD) score that is currently relied upon for prognosis in the US (Umbaugh et al., 2024).

However, while these biomarkers hold promise for prognosis, they are not currently available for clinical use and bringing them to market would be difficult. ALF is a relatively rare condition, so the market for a new ALF biomarker is small, and the process of shepherding new in vitro diagnostics (IVD) through the regulatory approval process can be costly. Although individual hospitals can create their own laboratory developed tests (LDTs) in-house, it is difficult to justify the cost to develop low volume LDTs in a busy clinical environment. Furthermore, the future of LDT regulation is unclear due to efforts by the US FDA to either duplicate or wrest control over such tests from the Clinical Laboratory Improvement Amendments (CLIA)-based oversight of the Centers for Medicare and Medicaid Services (Scott et al., 2013; Marzinke and Clarke, 2015; Genzen, 2019; Lin and Thomas, 2023; Marzinke et al., 2023; Saitman,
While large reference laboratories could offer these tests, those laboratories typically have long turnaround times that would not be ideal when a rapid decision regarding liver transplantation is needed. To overcome this issue, at least two alternative approaches to the straightforward development of new tests could be considered as we move further into the future of ALF prognostics discovery and development: First, we could focus on developing biomarkers with prognostic utility in ALF that also have uses in other, more common conditions that would justify the expense of development. Second, we could focus on repurposing existing biomarkers that are already widely used in clinical laboratories so no further development is required and clinical application can begin immediately. Fortunately, there are biomarkers that are already available clinically that have been shown to have prognostic value. These include α-fetoprotein (Schmidt and Dalhoff, 2005) and lactate dehydrogenase (LDH) (Vazquez et al., 2022; Price et al., 2023). These markers, alone or in combination with one another or with the MELD score, the King’s College Criteria, or the ALFSGPI, may thus be the most promising options for further clinical exploration.

CURRENT CHALLENGES, KNOWLEDGE GAPS, AND FUTURE DIRECTIONS

Mechanisms of injury that should be explored in more detail in humans include oxidative stress, the role of JNK, and the mechanism of nuclear DNA fragmentation. There is evidence for oxidative stress in APAP hepatotoxicity in human cell culture models. For example, dichlorofluorescein fluorescence has been observed in HepaRG cells treated with toxic concentrations of APAP (McGill et al., 2011), and JNK activation – which is mediated by oxidative stress – has been reported in freshly isolated primary human hepatocytes during APAP exposure (Xie et al., 2014). Thus, it is likely that oxidative stress occurs in the human liver after APAP overdose as well. However, the only evidence directly from humans is F$_2$-isoprostanes data, which is limiting. JNK activation is also underexplored. While, again, JNK activation has been observed in APAP hepatotoxicity in vitro and JNK inhibition protects against APAP in that model (Xie et al., 2014), there is currently no evidence that JNK plays a role in actual patients. This
would be easy to address, as one could simply compare western blots for phosphorylated JNK between APAP overdose patients with liver injury and patients with liver injury of other etiologies not involving JNK to determine if JNK has some role in humans. Finally, there have been no experiments, even \textit{in vitro}, to determine if the mechanism of nuclear DNA fragmentation is the same between mice and humans. Additional work in these areas may lead to not only new mechanistic information, but also potentially better prognostic biomarkers to guide patient care.

\textbf{CONCLUSIONS}

Overall, the development and application of translational, mechanistic biomarkers has revealed that the fundamental mechanisms of APAP hepatotoxicity in humans discovered over the last 50 years resemble those in preclinical models, particularly mice. Evidence of impaired BSP glutathionylation in APAP overdose patients and increased glutathione turnover in healthy volunteers taking therapeutic APAP doses is consistent with the glutathione depletion seen in experimental models. The detection of APAP-protein adducts in serum from humans after both APAP overdose and therapeutic APAP dosing clearly demonstrates that protein alkylation occurs in humans too. Importantly, elevated GLDH, mtDNA, nuclear DNA fragments, acylcarnitines, and CPS1 in APAP overdose patients indicates mitochondrial damage, and the fact that a few of these markers are even higher in non-survivors of APAP-induced ALF compared to survivors indicates that mitochondria drive the toxicity. Increased F$_2$-isoprostanes in APAP overdose patients also indicates oxidative stress, consistent with mitochondrial dysfunction, though more work using additional biomarkers is needed to verify the oxidative stress and explore it in greater detail. Finally, elevated HMGB1, the low proportion of K18 in the caspase-cleaved form, and the absence of caspase 3 activity in circulation of APAP overdose patients indicate that necrosis is the primary mode of cell death in APAP-induced liver injury in humans. Thus, the work begun in 1973 has stood the tests of time and human translation.
ACKNOWLEDGEMENTS

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DATA AVAILABILITY

Not applicable. This manuscript contains no new datasets.

CONFLICTS OF INTEREST

In the last 10 years, the author has consulted for Acetaminophen Toxicity Diagnostics, LLC, Alkermes, GlaxoSmithKline, and REMD. The author has also served as an expert witness for Butler Snow, LLP. These organizations had no role of any kind in the conception of this review, nor in the drafting, editing, or submission of the manuscript.

AUTHOR CONTRIBUTIONS

MRM was solely responsible for the conceptualization, drafting, and editing of this manuscript.

REFERENCES


TABLES

Table 1. Translational, mechanistic biomarker studies in humans.

<table>
<thead>
<tr>
<th>Mechanistic Event</th>
<th>Human Biomarker Evidence</th>
<th>Selected citation(s)</th>
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<tbody>
<tr>
<td>Glutathione depletion</td>
<td>• APAP-glutathione derivatives in urine and serum</td>
<td>• Jagenburg and Toczko, 1964</td>
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<td></td>
<td>• Prolonged BSP clearance</td>
<td>• Davis et al. 1975</td>
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<td></td>
<td>• Radiolabeled APAP-glutathione from radiolabeled cysteine</td>
<td>• Lauterburg and Mitchell, 1987</td>
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<tr>
<td>Protein alkylation</td>
<td>• Serum APAP-protein adducts</td>
<td>• Hinson et al., 1990; numerous other studies</td>
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<tr>
<td>Mitochondrial damage</td>
<td>• Elevated GLDH, mtDNA, and nuclear DNA fragments in serum</td>
<td>• McGill, Sharpe, et al., 2012; McGill, Staggs, et al., 2014</td>
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<td></td>
<td>• Elevated CPS1 in serum</td>
<td>• Weerasinghe et al., 2014; Kwan et al., 2023</td>
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<td></td>
<td>• Elevated plasma acylcarnitines</td>
<td>• McGill, Li, et al., 2014; Bhattacharyya et al., 2014</td>
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<td>Oxidative stress</td>
<td>• F₂-isoprostanes in urine</td>
<td>• Morrow et al., 1993; Delanty et al., 1996</td>
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<td>Nuclear DNA fragmentation</td>
<td>• Elevated DNA fragments / histones in serum</td>
<td>• Craig et al., 2011; McGill, Sharpe, et al., 2012; McGill, Staggs, et al., 2014; Laurent et al., 2020</td>
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<td>Necrosis</td>
<td>• Low proportion of ccK18 in circulation</td>
<td>• Volkmann et al., 2008; Craig et al., 2011</td>
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<td></td>
<td>• Lack of caspase 3 activity in serum</td>
<td>• McGill, Sharpe, et al., 2012</td>
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<td></td>
<td>• Total HMGB1 elevation in circulation</td>
<td>• Craig et al., 2011; Dear et al., 2018</td>
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<td>Accumulation of toxic bile acids</td>
<td>• Elevated circulating bile acids</td>
<td>• Woolbright et al., 2014; James et al., 2015; Luo et al., 2018; Ghallab et al., 2022</td>
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<td>Inflammation</td>
<td>• Various cytokines (namely, IL-6,</td>
<td>• James et al., 2001, 2004, 2005; Williams et al.,</td>
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<td>Role</td>
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<td>Lect2</td>
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<td>CXCL14</td>
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2003; Woolbright et al., 2022; Etc.

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<th>Role</th>
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**Regeneration**

- Liver function tests (bilirubin, prothrombin time)
- AFP
- PA
- Regeneration-associated miRNAs

- Schmidt and Dalhoff, 2005
- Lutkewitte et al., 2018
- Salehi et al., 2013, 2020; John et al., 2014; Tavabie et al., 2021 (Salehi et al., 2013, 2020; John et al., 2014; Tavabie et al., 2021)
FIGURES

Figure 1. Schematic of APAP hepatotoxicity mechanism based on biomarkers. After acetaminophen (APAP) overdose, APAP is converted to the reactive intermediate N-acetyl-p-benzoquinone imine (NAPQI), which binds to and depletes glutathione in the liver. The NAPQI also binds to proteins, forming APAP-protein adducts. Targeting of mitochondria leads to mitochondrial damage and dysfunction, with the resulting accumulation of long-chain acylcarnitines due to impaired lipid oxidation as well as release of mitochondrial proteins (pink dots), such as glutamate dehydrogenase (GLDH) and carbamoyl phosphate synthetase 1 (CPS1), and mitochondrial DNA (mtDNA) into the cytosol. The mitochondrial endonucleases apoptosis-inducing factor (AIF) and Endonuclease G are also released, then translocate to the nucleus and fragment nuclear DNA. Upon necrosis, with loss of cell membrane integrity, all of these proteins and other molecules are released into the blood where they can be measured along with high mobility group box 1 (HMGB1) protein and keratin 18 variants. Bile acids also accumulate and may contribute to APAP hepatotoxicity too.

Figure 2. Inflammation and regeneration biomarkers. The initial injury after APAP overdose leads to release of damage-associated molecular patterns (DAMPs; e.g. nuclear DNA, mtDNA) and chemokines (e.g. CXCL14, Lect2) that can recruit and/or activate inflammatory cells, including Kupffer cells (KCs), infiltrating macrophages, and neutrophils (NTs). Some of these activated cells (e.g. KCs) then produce cytokines which propagate the inflammation. Around the same time, there is activation of cell proliferation signaling via Wnt ligands and other growth receptor ligands, with accumulation of β-catenin which then promotes transcription of pro-proliferative genes. Phosphatidic acid (PA) also relieves inhibition of β-catenin signaling to support these processes. As the liver regenerates, liver function recovers, leading to normalization of serum bilirubin and of coagulation. α-fetoprotein is highly expressed by these proliferating hepatocytes and can be measured in circulation. PA can also be measured in circulation, though the mechanism of release or secretion is currently unclear. In addition, regeneration-associated microRNAs (miRNAs) are released.
Figure 2.