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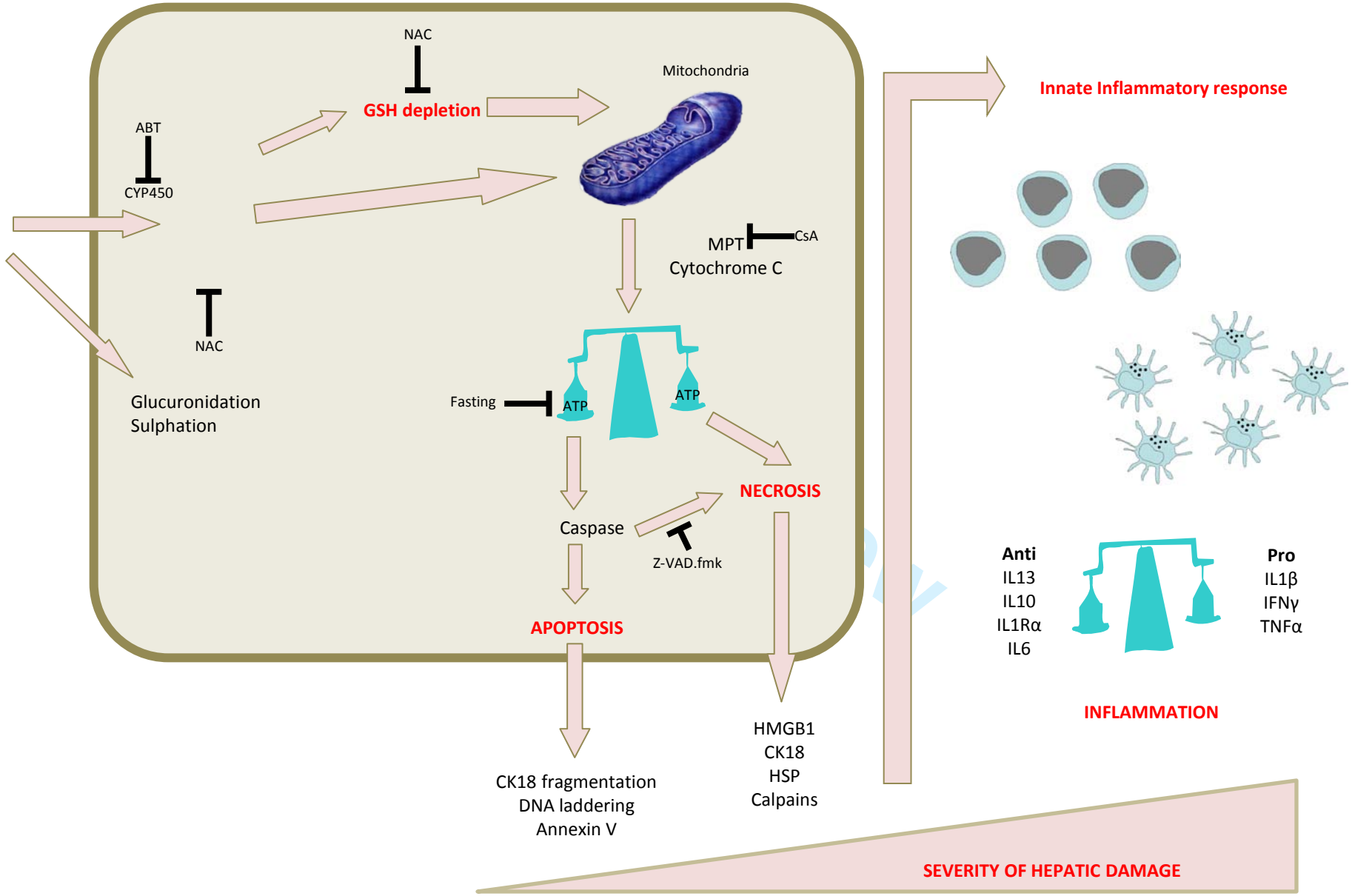
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The Potential of Cytokines as Safety Biomarkers for Drug-Induced Liver Injury

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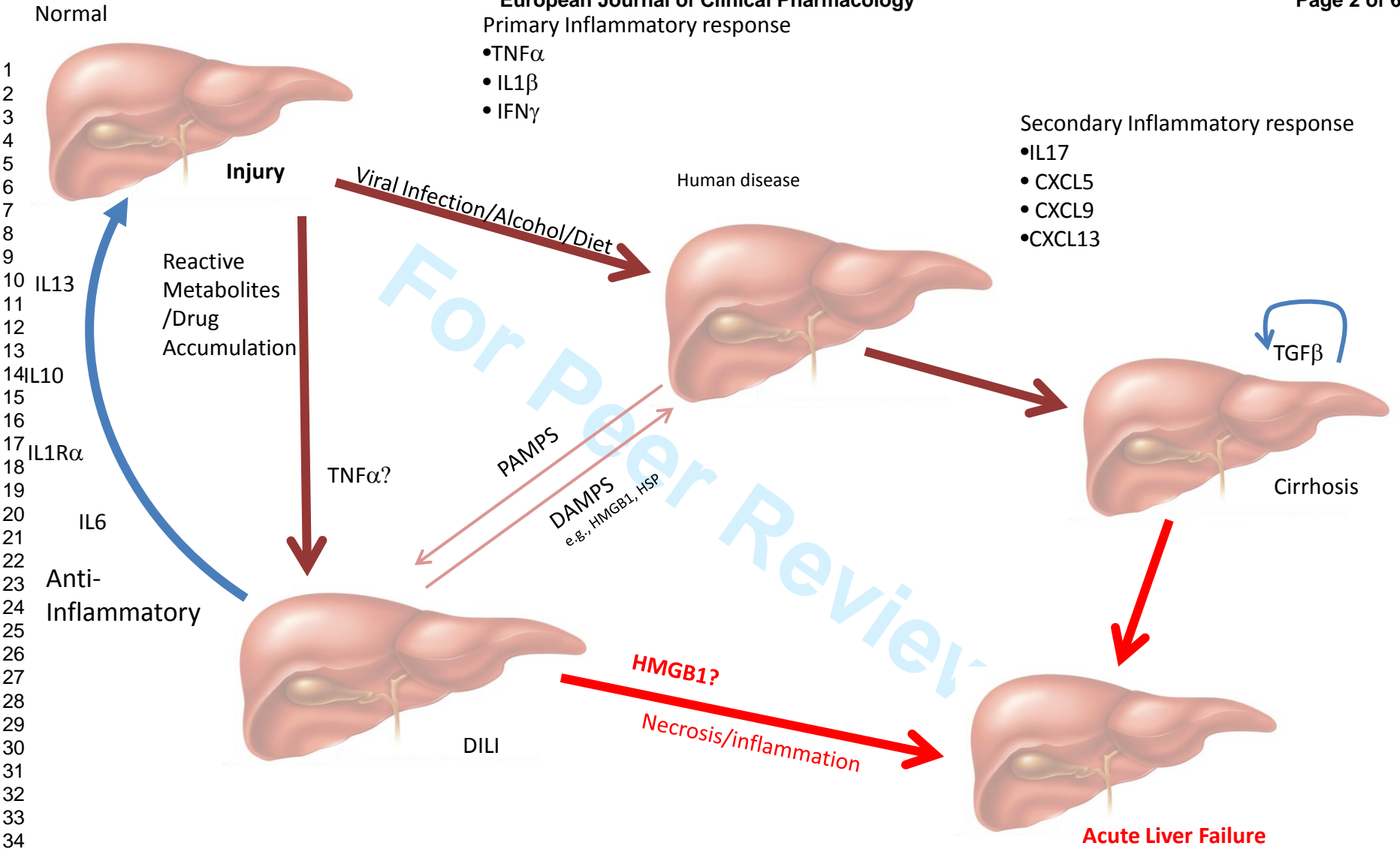


Primary Inflammatory response

- TNF α
- IL1 β
- IFN γ

Secondary Inflammatory response

- IL17
- CXCL5
- CXCL9
- CXCL13



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The Potential of Cytokines as Safety Biomarkers for Drug-Induced Liver Injury

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Abstract

Drug-induced liver injury (DILI) is an event that has a detrimental impact on drug development and patient safety, therefore the identification of novel biomarkers that are not only sensitive and specific to the liver would have great benefit. Inflammation is known to be associated with human cases of DILI and given the role of cytokines in modulating the inflammatory response changes in cytokine expression patterns certainly show promise as potential biomarkers of DILI. Cytokines are interesting candidates for novel biomarkers as they are relatively accessible (by blood sampling) and accurately quantifiable. In particular, recent interest has developed in mechanism, rather than tissue, specific biomarkers. However, without fully understanding the role of inflammation in DILI, and the role of cytokines in modulating the inflammatory response, they may be limited in their use, being either diagnostic of the type of injury that has occurred and/or prognostic of outcome (recovery from DILI, cirrhosis, acute liver failure). Intracellular components released by damaged hepatocytes, although inaccessible and currently difficult to quantify, may be better biomarkers for the prognosis of severity of injury. In both cases there is a pressing need for the development and validation of assays sensitive enough and with a sufficient dynamic range to detect changes upon drug treatment. Although promising candidates are appearing in the literature much remains to be done to understand the role of inflammation in DILI and the role that a given cytokine has in the inflammatory cascade associated with DILI before cytokines are viewed as biomarkers for DILI.

Key words

Cytokine, inflammation, pharmaceutical, biomarker, drug-induced liver injury

Introduction

Drug Induced Liver Injury (DILI) is an adverse event that leads to regulatory action on drugs culminating in serious instances of withdrawal from the market. At a regulatory level hepatotoxicity is the single most frequent reason for removing approved medications from the market. Between 1975 and 1999, 548 new drugs were approved by the FDA of which 10 received a 'black box warning' for potential hepatotoxicity and an additional four were withdrawn from the market [1]. The population incidence of drug-induced liver injury is unknown as most events are often inaccurately classified [2-4]. Yet DILI is the most frequent cause of acute liver failure among those under consideration of liver transplantation in US [5], in the UK alone paracetamol (acetaminophen, APAP) overdose accounts for 200-500 deaths and 20-40 liver transplants annually [6]. In drug development animal studies detect approximately half of compounds exhibiting hepatotoxicity in man [7], whereas *in vitro* human hepatocyte testing detects 50-60% of drugs that can cause severe liver injury in man, including some not detected by animal testing [8]. So although current testing methodologies are capable of detecting some hepatotoxins in drug development there are no currently available preclinical tests that detect the potential for serious or idiosyncratic human hepatotoxicity with combined high sensitivity and specificity.

If drugs capable of causing serious DILI at a very low frequency are identified, there is a need for reliable biomarkers that could be used to predict which individuals are susceptible to develop DILI from that drug either before drug exposure, or at a very early time after drug treatment has begun (e.g., assays of substances in blood or urine). Ideally, these biomarkers would discriminate between individuals susceptible to, those capable of adapting to and those that can tolerate DILI. Upon their validation, such biomarkers could facilitate approval of drugs that would otherwise not be possible, by excluding identified susceptible people who should never be exposed to that particular drug [9].

To be able to identify novel biomarkers for evaluation of new chemical entities or determination of individual patient susceptibility requires a thorough understanding of the mechanisms involved in DILI. Although the pathogenesis of DILI is not fully understood, studies of mechanisms in animal models can be used to split DILI into a two stage process. Stage I often involves either excessive drug accumulation, biotransformation and metabolic activation, which can be followed by covalent binding and transport when bioinactivation processes become saturated or immobilized resulting in chemical stress which in turn leads to in a cellular effect e.g., mitochondrial dysfunction (Figure 1). The identification of biomarkers at this stage is difficult unless a 'drug

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3 related-metabolite' or a modified protein is secreted into the blood stream and/or urine at levels
4 that can be detected. Often the earliest changes observed in safety screening are histopathological
5 and therefore not practical for use as a biomarker in humans. Stage II represents subsequent
6 processes of adaptation or failure of response to modification of essential cellular processes [10]
7 which may be partially mediated by liver nonparenchymal cells, Kupffer cells and/or infiltrated
8 macrophages [11-13]. The changes within the liver at this stage of DILI are more amenable to
9 biomarker discovery. Injured hepatocytes release factors that are chemoattractant and subsequently
10 activate non-parenchymal cells potentially compounding cellular damage by releasing cytotoxic
11 mediators. Although the mediators for progression of DILI have not yet been absolutely defined,
12 recent evidence supports the hypothesis that the types of cytokine produced in response to the
13 initial insult influence the severity of the toxicity [14]. Can we use human liver disease and animal
14 models, where inflammation is known to play a role to better understand the role of inflammation in
15 DILI and in so doing identify novel biomarkers for DILI or identify potential strategies for therapeutic
16 intervention.
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29 **Clinical features of DILI and current assessment**

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31 Due to complexity within the structure and the function of the liver, clinical symptoms of
32 drug-induced hepatotoxicity may take many forms. DILI may mimic various forms of naturally
33 occurring liver disease, reviewed previously [15]. The assessment of DILI is primarily based on the
34 relatively non-invasive quantification of blood protein biomarkers and hallmarks of hepatic
35 biochemistry. The clinical manifestations of DILI range from mild asymptomatic biochemical changes
36 to fulminant hepatic failure. However, the most useful form of differentiation of hepatic pathology is
37 between cholestatic or hepatocellular injury [15] which is based on the differential quantification of
38 blood hepatic enzymes (Table 1).
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Hepatic blood enzymes are roughly grouped in to hepatocellular markers, such as serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and the cholestatic markers alkaline phosphatase (ALP), and γ -glutamyltransferase (GGT). These enzymes are most often elevated in patients with liver disease and may reflect liver injury. However, they are present in tissues throughout the body and often physicians are faced with the problem of a wide differential diagnosis with low specificity. Currently there are no pathognomonic indicators of DILI; even liver biopsy is not diagnostic, while confounding factors such as viral hepatitis or congestive heart failure may not be recognized in the setting of hepatic illness. In certain patient groups, such as diabetic or obese

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3 persons, for example, liver test abnormalities are common, and other medical conditions such as
4 coronary artery disease and renal failure may confound clear identification of affected patients.
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9 The sensitivity of these tests is questionable. In clinical trials mild elevations of ALT or AST
10 are used as markers of DILI. However, the thresholds used are not consistent between trials in
11 defining DILI: variously placed at twice, thrice, five times or even ten times the upper limit of the
12 normal reference range, but are typically below 40 IU/L. ALT is thought to be a more specific
13 indicator than AST, but normal values vary greatly between laboratories [16]. Despite the association
14 between greatly elevated ALT levels and hepatocellular diseases, the absolute height of the ALT
15 elevation does not correlate with the extent of liver cell damage [17]. Transient serum enzyme
16 activity elevations, however high, are common and can be confusing. The physician is never sure
17 whether they would translate into potentially serious clinical symptoms of hepatic illness.
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26 The use of the current normal range may lead to an underestimated prevalence of liver
27 disease [18]. Because the enzyme concentration in a population forms a continuous distribution, the
28 cut-off concentration that discriminates between healthy and diseased livers is not clearly defined
29 [19]. Indeed several studies have recently questioned whether previously established values to
30 define normal ALT range are accurate and have suggested that the upper limit of normal should be
31 revised [20]. Various scoring tools have been developed to assess causality (RUCAM) usually
32 involving complex calculation of probability based on ALT, ALP, GGT ratios weighed against time of
33 onset, age, alcohol use, viral hepatitis infections, biliary tract disease, or hepatic ischemia. The
34 validation, accuracy and scientific basis of these tools has been problematic. Powerful computing
35 tools and sophisticated Bayesian algorithms [21] have been used but these are not readily available
36 for the clinician at the bedside.
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46 Bilirubin cannot be reliably used as a marker of early liver injury. Once again the threshold is
47 ill-defined and once arbitrarily set can be a late marker of liver injury. Many disease processes and
48 inherited disorders such as Gilbert's syndrome may lead to elevated levels of bilirubin not related to
49 DILI and may falsely raise alarm bells to the treating physician. The appearance of jaundice is often a
50 late marker of disease. Another commonly used test, the blood prothrombin time (or its derivative
51 Internationalized Ratio, INR) may be useful as a liver function test (of protein synthesis), however,
52 individual laboratories have to determine their own reference values. In paracetamol overdose the
53 INR can be used prognostically especially on day 2 or 3 to determine deterioration or improvement.
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60 However, this too is a late indicator of liver dysfunction and initially may not predict which patients

are likely to deteriorate. Arterial blood lactate measurement rapidly and accurately identifies patients who will die from paracetamol induced acute liver failure. Its use could improve the speed and accuracy of selection of appropriate candidates for transplantation.

Consequently there is no specific non-invasive diagnosis, treatment or prevention of DILI except for the early withdrawal of a drug treatment to the individual in the case of suspected DILI. Currently, the monitoring of the parameters described in Table 1 represents the best indication of hepatic injury. Therefore newer, rapid and reflective non-invasive biomarkers are required that are not only sensitive for hepatic injury but also provide insight into the mechanism of pathology. There are a number of potential biomarkers reflective of hepatocellular damage which may offer superiority over ALT determination [22]. However, there are a limited number of candidates reflective of other pathologies observed during DILI, namely hepatitis, steatosis, fibrosis and cholestasis. Therefore, a comprehensive understanding of the mechanisms that lead to the development of these conditions is required to reveal potential biomarkers specific for established liver pathologies.

Table 1: Standard panel of currently used blood clinical chemistry parameters to assess hepatic function and injury. Taken from [23].

| Biomarker | Injury type | Normal reference range |
|--------------------------------------|------------------|----------------------------------|
| Alanine aminotransferase (ALT) | Hepatocellular | 5-40 IU/L |
| Aspartate aminotransferase (AST) | Hepatocellular | 10-40 IU/L |
| Alkaline phosphatase (AP) | Cholestatic | 30-120 IU/L |
| Gamma glutamyl-transpeptidase (GGT) | Cholestatic | 0-51 IU/L |
| Total bilirubin | Hepatic function | 2-14 $\mu\text{mol/L}$ |
| Direct (conjugated) bilirubin | Hepatic function | 0-4 $\mu\text{mol/L}$ |
| Pro-thrombin time (coagulation test) | Hepatic function | Local laboratory reference value |

Biomarkers of DILI

Biomarkers are defined as an objectively measured change in either DNA, protein or metabolite levels that is reflective of a normal biological process, pathological condition or correlates with the pharmacological action of a therapeutic agent [23, 24]. The ideal DILI biomarker would have the following characteristics:

- Reflective of the actual hepatic injury/repair status
- Rapidly assayed and can be assayed in any laboratory
- Non-invasive, i.e. present in the blood or urine compartment
- Transferable across *in vitro*, *in vivo* and clinical systems to further inform and accelerate drug safety screening
- Able to precede the onset of massive hepatic injury – secreted markers rather than leakage
- Of a low base-line variability in control samples and normal population
- Accurately identifiable and quantifiable with a suitable dynamic range to detect upon drug treatment

The current clinical standard for assessing inflammation by a blood borne biomarker is through the quantification of C-reactive protein (CRP). CRP is an acute-phase protein which is produced rapidly in response to inflammation and infection. The normal physiological role of CRP is to activate the complement system by C1q by binding phosphocholine expressed on the surface of dead / dying cell (and bacteria) which enhances phagocytosis by macrophages. CRP is synthesised in the liver, therefore CRP holds little utility as a marker of inflammation when hepatic dysfunction or DILI is suspected [25].

Taking the characteristics above in to account, cytokines as potential novel biomarkers of DILI, in particular idiosyncratic DILI, represent promising candidates. With underlying inflammation thought to be a contributing factor for the onset of idiosyncratic DILI [26-29], the quantification of cytokines as potential predictive markers of patient susceptibility holds promise. However, the large variation observed in base-line levels in normal populations renders the establishment of reference values to assay meaningful elevations difficult. There are many established and robust commercially available ELISA assays utilising non-invasive biofluids for the accurate absolute quantification of cytokines. Ease of assay use, the presence in a non-invasive biofluid and the species cross reactivity of antibodies used in ELISAs also aids the translation between pre-clinical and clinical studies. These factors represent the main advantage of cytokines as biomarkers of DILI [30]. In healthy volunteers and control pre-clinical test species with tightly controlled diets and environments cytokine

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3 elevations often exhibit a large dynamic range upon drug treatment. However, it is important to
4 note that added sensitivity and dynamic range may give rise to an increase in false positives, which
5 in turn may lead to the failure to progress an otherwise safe candidate in development or the
6 withdrawal of potential efficacious drug treatment.
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12 When assessing novel candidates as biomarkers, it is important to have a comprehensive
13 understanding of biomarker kinetics and distribution. The active secretion of cytokines from cells
14 present in the liver and at distant sites indicates that blood elevations may occur prior to cell death
15 or organ dysfunction in response to mild, non-toxic cell stress. Moreover, given the short half life of
16 cytokines, they return to background levels rapidly and therefore may also represent markers of
17 reversible hepatic damage, such as that shown recently for High Mobility Group Box-1 protein
18 (HMGB-1) in a mouse model of acetaminophen hepatotoxicity [31]. Focussing upon the kinetics of
19 blood biomarker appearance and a particular signature pattern of cytokine expression may give a
20 real insight into hepatic function and toxicity state. However, caution should be taken in the
21 interpretation of multiple biomarker kinetics because many of these biomarkers are not organ
22 specific. Novel, possibly non-specific, biomarkers of damage should always be assessed alongside the
23 gold standard biomarkers for that organ, e.g. ALT/AST for hepatic damage. The correlation between
24 pro-inflammatory cytokine level changes in blood (increase/decrease) with the extent of liver
25 damage or established biomarkers is not fully known and warrants further investigation.
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38 **The Role of inflammatory Cells and Cytokines in Liver Disease**

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40 Given that cytokines have several of the features required for novel, mechanism biomarkers
41 what data is available in terms of human disease and liver injury for their use as a biomarker in drug
42 safety. Human liver disease takes many forms and can result from viral hepatitis [31], obesity (fatty
43 liver disease; [32]), alcohol abuse (fatty liver disease, alcoholic hepatitis, cirrhosis [33]), genetics,
44 autoimmune disorders (primary biliary cirrhosis, primary sclerosing cholangitis and autoimmune
45 hepatitis [34]). As the inflammatory component has been best described in the cases of viral hepatitis
46 and alcoholic liver disease we focus on the common themes in inflammation associated with these
47 two types of liver injury.
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55 **Viral Hepatitis**

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57 At all stages of viral hepatitis infection chemokines are key regulators of immunity and
58 inflammation functioning within cytokine cascades that regulate the immune response to the virus.
59 However in chronic infection their persistent expression can drive chronic inflammation leading to
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3 liver injury and cirrhosis [36]. The Hepatitis B Virus (HBV) itself is non-cytopathic but immune
4 mediated liver damage often occurs in patients with both acute and chronic HBV infection. Such
5 damage has been attributed to the killing of infected hepatocytes by virus specific toxic CD8+ T cells
6 [37, 38]. There is increasing evidence to suggest that a non-HBV-specific inflammatory infiltration
7 into the liver is likely responsible for hepatic pathology in patients with chronic hepatitis B [39, 40].
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14 Upon infection with Hepatitis C Virus (HCV) hepatocytes secrete interferon (IFN) and down
15 regulate RNA translation as a response to infection before innate immune cells, including
16 macrophages, dendritic cells (DC), natural killer cells and natural killer T cells are activated to amplify
17 secretion of type-1 IFNs and IFN-response genes. Activation of Toll-like receptors, triggers
18 chemokine secretion which amplifies leukocyte recruitment [41, 42]. The activation of innate
19 immunity by the uptake and processing of viral antigen by DCs is followed by the migration of DCs to
20 the lymph nodes where they present viral antigen to naive T cells leading to their activation and the
21 development of an adaptive immune response [43]. Two types of DCs have been characterised in
22 the response to HCV infection of hepatocytes, myeloid dendritic cells (mDCs) and plasmacytoid
23 dendritic cells (pDCs). pDCs secrete type-1 IFN, prime T helper 1 (Th1) responses and activate
24 cascades of chemokine secretion in HCV infection [44]. mDCs prime Th1 responses via interleukin 12
25 but produce little type-1 IFN [44, 45]. In the early phases of viral infection chemokines particularly
26 CXCL8, CXCL16, CCL2 and CCL3 promote recruitment of innate immune cells to the liver, including
27 DCs which then initiate the adaptive immune response and it is likely that similar events shape the
28 response to HCV infection [46-47].
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41 TH1 immune responses dominate in the HCV-infected liver (Shields et al., 1999) and
42 intrahepatic T cells express chemokine receptors associated with Th1 responses eg., CXCR3 [49].
43 Hepatic CXCR3 ligands are increased in many liver diseases and this suggests that they play a role in
44 effector cell recruitment to the inflamed liver [50]. Expression of CXCR3 is closely linked to Th1
45 function and its ligands CXCL9, CXCL10 and CXCL11 are induced by the TH1 cytokines IFN γ and TNF α
46 [51, 52]. In contrast to HCV, HBV infections are characterised by increased numbers of TH17 cells
47 producing a cocktail of cytokines of which IL17A is characterised as a major effector cytokine [53].
48 IL17A can mobilise, recruit and activate neutrophils leading to massive tissue inflammation and
49 promote the progression of autoimmune disease [54]. Peripheral and intrahepatic TH17 cells are
50 preferentially increased in patients with chronic HBV infections, which may activate mDCs and
51 monocytes to release inflammatory cytokines during chronic HBV infection [55].
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Viral clearance is associated with a vigorous CD4+ and CD8+ T cell response again with Th1 responses dominating. Regulatory T cells (Tregs) have been found in the HCV-infected liver and appear to have a dualistic effect, they suppress HCV-specific CD8+ T cells and promote viral replication however they also appear to suppress collateral inflammatory damage to reduce liver injury in chronic infection [56, 57]. Successful antiviral therapy is associated with an increase in circulating CXCR3+CD8+ T cells and a reduction in CXCL10 and CXCL9 levels in blood [52, 58, 59]. Interestingly the vasculitic lesions of cryoglobulinemia in nerves and skin are characterised by upregulation of CCL3, CCL4 and CXCL10 and infiltration by CXCR3+ Th1 cells and CCR5+ monocytes. Thus the same pro-inflammatory chemokines are implicated in vasculitic complications of globuliemia and hepatitis [60].

Alcohol Liver Disease

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Factors such as gender, age, nationality, weight and health can affect how a person's liver metabolizes alcohol. When the liver has too much alcohol to handle, normal liver function may be interrupted leading to a chemical imbalance. If the liver is required to detoxify alcohol continuously, liver cells may be destroyed or altered resulting in fat deposits (fatty liver) and more seriously, either inflammation (alcoholic hepatitis) and/or permanent scarring (cirrhosis). Liver cancer can also result from alcohol induced liver disease. It is increasingly evident that chronic inflammation represents the driving force in the evolution of alcohol-induced liver injury to steato-hepatitis and cirrhosis. Accordingly, the hepatic expression of pro-inflammatory cytokines precedes the development of histologic signs of necro-inflammation in alcohol fed rats [61], while steato-hepatitis is associated with leucocyte focal infiltration and elevated circulating levels cytokines/chemokines such as TNF α , IL1, IL6, IL8/CINC and macrophage chemotactic protein-1 (MCP1) [62-65].

Innate and adaptive immunity in alcohol liver disease

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The interaction between alcohol and hepatic inflammation is complex. Early studies proposed the involvement of innate immunity with the increased translocation of gut-derived endotoxins to the portal circulation being recognised by Kupffer cells via the toll-like receptor-4 (TLR4 [63, 66]). Ethanol itself has been found to enhance the capacity of Kupffer cells to respond to pro-inflammatory stimuli via the up regulation of Toll-like receptors [67] and the production of pro-inflammatory cytokines and chemokines by modulation of signalling pathways such as nuclear transcription factor κ B (NF κ B), early growth response-1 (ERG1) as well as JNK, ERK and p38 protein kinases [65, 68-70].

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Histologic examination of liver biopsies from patients with alcoholic hepatitis reveals the presence of CD4+ and CD8+ T-lymphocytes [71]. The influence of adaptive immunity in the response to alcohol is supported by the predominance of Th1 cytokines (high TNF α , INF γ , IL1) in peripheral blood T-cells isolated from active drinkers with or without ALD [72]. Activated liver-infiltrating IL17-producing T Helper (TH17) cells are also responsible for neutrophil recruitment into the liver [73]. Furthermore serum IL17 levels are increased and serve as a marker of the severity of acute hepatic injury [74]. Evidence is accumulating to suggest that TH17 lymphocytes have an important role in driving inflammation during the development of other chronic inflammatory and autoimmune diseases including inflammatory bowel disease, psoriasis rheumatoid arthritis and multiple sclerosis [75] as well as viral hepatitis as has been discussed.

Alcohol may also have an effect on the immune system by effects on osteopontin, leptin and adiponectin. Osteopontin is a cytokine that enhances the pro-inflammatory Th1 response and the livers of alcohol fed rats have been found to have an associated increase in osteopontin levels [76] while patients with alcoholic hepatitis have higher levels of osteopontin mRNA in their livers compared to patients with fatty liver only [77]. Leptin has been shown to have a pro-inflammatory function while adiponectin counters pro-inflammatory cytokine production [78]. Alcohol appears to have opposite effects on these adipokines, patients with ALD have increased serum leptin [79] whereas chronic ethanol feeding decreases adiponectin secretion in the early phase of alcohol injury in rodents [80, 81].

Signal transduction pathways

In response to alcohol several different signal transduction pathways are activated. Transcription factors regulating fatty acid metabolism including sterol regulatory binding protein (SREBP) and peroxisomal proliferating factor α (PPAR α) play a pivotal role in early alcoholic liver injury [82]. One of the most well studied transcription factors is NF κ B which has a pivotal role in controlling the expression of pro-inflammatory cytokines in monocytes and macrophages. However the expression of NF κ B displayed a surprising response to ethanol treatment depending on whether the treatment is acute or chronic. Differences have been noted in the ability of acute and chronic alcohol exposure to modulate monocyte/macrophage activation. Acute alcohol exposure is associated with LPS-induced anti-inflammatory effects in monocytic cells in humans both *in vivo* and *in vitro* [83-85]. By contrast chronic alcohol induces the expression of TNF α in human and mouse models [69, 86, 87]. This switch between anti-inflammatory and pro-inflammatory responses is linked with changes in IL-1R-associated kinase-monocyte (IRAK-M), a negative regulator of LPS

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3 signalling and leads to decreased downstream activation of LPS signalling cascade culminating in
4 impaired NFκB DNA binding and transactivation and finally TNFα production. In contrast chronic
5 alcohol exposure switches the anti-inflammatory to a pro-inflammatory response by decreasing
6 IRAK-M and increasing IRAK-1 and IKK kinase activation followed by enhanced NFκB DNA binding,
7 transactivation and ERK kinase activation and TNFα production [88]. Other transcription factors such
8 as Activator Protein-1 (AP1), PPARγ and Egr1 have also been shown to be modulated by exposure to
9 alcohol [89, 90, 91].
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17 **Animal models of DILI associated inflammation**

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19 There is a lot of data to support the role of inflammation in the progression of human liver
20 disease as has been described for viral hepatitis and alcohol liver disease. Currently sufficient data to
21 support an unequivocal role for inflammation in DILI is lacking and in many cases mechanistic details
22 are unknown. Evidence to support the hypothesis that DILI triggers the activation of the innate
23 immune system within the liver has been obtained from experimental *in vivo* models. The
24 advantages of investigating inflammation in animal models of DILI are the stable genetic, molecular
25 and immunological backgrounds of inbred strains. This lack of variation enables a more complete
26 exploration and determination of possible inflammatory mechanisms contributing to the overall
27 pathophysiology. However, although animal models are useful tools with which to investigate DILI
28 and the involvement of inflammation caution must be shown in the translation of these results to
29 human DILI.
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40 **Inflammation in an acetaminophen (APAP) model of DILI**

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42 A widely studied model of DILI has been APAP-induced liver injury (ALI) in mice (Table 2).
43 Evidence suggests that the initial hepatocellular damage caused by NAPQI may lead to the release of
44 cellular contents known as damage associated molecular patterns (DAMPs). These include HMGB-1
45 or Heat shock proteins (HSP) which act as signals which can activate cells of the innate immune
46 system which reside in the liver. This in turn leads to hepatic infiltration of inflammatory cells which
47 may contribute to the progression of liver injury by producing pro-inflammatory mediators such as
48 cytokines and chemokines as well as reactive oxygen and nitrogen species (Figure 1, [92]).
49 Neutrophils accumulating in necrotic areas of the liver after APAP overdose were first recognised by
50 Mitchell et al. [93] but the relevance of this accumulation to the pathophysiology is still unclear [94].
51 A study in C3Heb/FeJ mice revealed that neutrophils accumulate in the liver shortly after hepatic
52 injury but there was no evidence of neutrophil activation. Additionally, antibodies targeted at these
53 cells did not protect against ALI [95]. In a rat model of ALI, a significant reduction in neutrophil
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3 accumulation showed no protection of liver injury [96]. These findings are in contrast to other
4 models in which neutrophils are involved in the course of injury where pre-treatment with an anti-
5 neutrophil antiserum attenuated AILI in rats [97].
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10 Other studies have demonstrated that mice treated with gadolinium chloride to inactivate KCs
11 [98, 99] and liposome/chlodronate to deplete KCs [99, 100] are protected against APAP toxicity.
12 Though reports suggest that KC depletion confers protection at early times after APAP treatment
13 [100]but can lead to more severe injury at later time points [99]. The use of an anti-NK1.1
14 monoclonal antibody to deplete NK and NKT cells significantly protected mice from APAP toxicity
15 probably due to the reduction in the levels of the pro-inflammatory cytokine IFN- γ [101]. However as
16 has been observed with KC depletion, there are conflicting reports suggesting that NK/NKT cells play
17 little role in the overall outcome of liver injury in the mouse model of APAP hepatotoxicity [102,
18 103]. This has been supported by a recent study that suggested NK and NKT cells only contributed to
19 liver injury when dimethyl sulfoxide (DMSO) was used to solubilise APAP [103]. Given the conflicting
20 data available on neutrophils and KCs it is clear that more studies are required to determine their
21 precise role in DILI.
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33 The involvement of various pro-inflammatory mediators such as TNF- α [104-106], IL-1ra [107],
34 IFN- γ [101, 108], in promoting tissue damage have been demonstrated whereas other cytokines
35 such as IL-10 and IL-4 [109] have been found to be hepatoprotective in models of APAP
36 hepatotoxicity. Receptors involved in promoting an inflammatory response such as IL-1 receptor
37 [106], p55 TNF- α receptor-1 [105], Toll-like receptors TLR4 and -9 [110, 111] and receptor for
38 advanced glycation end-products (RAGE) [112] have been found to be important in promoting an
39 inflammatory response and subsequent pathogenesis.
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Table 2: Summary of inflammatory models used in APAP-induced hepatotoxicity

| References | Inflammatory model(s) | Reported effect |
|------------|--|---|
| [99, 100] | KC depletion | Protection against liver injury at early time points, more susceptible at later time points |
| [113] | Neutrophil depletion | Significantly protected against liver injury |
| [101] | NK/NKT cell depletion | Significantly protected against liver injury |
| [106] | Anti-TNF- α /IL-1- α /IL-1ra | Increased susceptibility to liver injury |
| [105] | Anti-TNF- α , TNF-p55 receptor 1 ^{-/-} mice | Reduced mortality and liver injury |
| [120] | TNFR1 ^{-/-} mice | Increased susceptibility to liver injury |
| [108] | IFN- γ ^{-/-} mice | Significantly protected against liver injury |
| [117] | IL-6 ^{-/-} mice | Increased susceptibility to liver injury |
| [109] | IL-10/4/6 ^{-/-} mice | Significantly protected against liver injury |
| | IL-10/4 ^{-/-} mice | Increased susceptibility to liver injury |
| [118] | IL-13 ^{-/-} mice | Increased susceptibility to liver injury |
| [111] | Anti-TLR9, TLR9 ^{-/-} , Nalp3 ^{-/-} mice | Reduced mortality and liver injury |
| [112] | Soluble RAGE | Reduced mortality and liver injury |
| [115] | LPS | Increased susceptibility to liver injury |

Comparison of the inflammatory response in animal models of acetaminophen-induced liver injury

Several different models of DILI in which inflammation has been modulated are being investigated in an attempt to dissect the processes of inflammation from those on DILI. NK and NKT cells were found to play a critical role in the severity and progression of AILI (500mg/kg, i.p) by secretion of cytokines and recruitment of inflammatory cells (mainly neutrophils) into the liver [101, 113]. An increase of hepatic neutrophil accumulation as early as 4-6 hours with a significant 3-fold increase by 24 hours was observed in APAP-treated mice with an associated increase in mRNA levels for cytokines TNF- α and IFN- γ [101]. Depletion of NK and NKT cells (using an anti-NK/NKT cell antibody or an anti-neutrophil antibody) inhibited liver injury with markedly reduced accumulation of neutrophils in the liver. Subsequent depletion of neutrophils also significantly protected mice

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3 against AILI. However, in a recently published fed CD-1 mouse model of AILI, no evidence of hepatic
4 inflammatory cell infiltration was observed over a 24 hour time course [114].
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9 Non-toxic doses of APAP (100-400mg/kg, i.p) administered 2 hours after a dose of LPS (44×10^6
10 EU/kg) to fasted C57BL6 mice have been shown to produce a leftward shift of the dose-response
11 curve for AILI [115]. Application of LPS led to significantly greater TNF- α production than APAP alone.
12 With APAP treatment alone there was a modest, sustained accumulation of neutrophils from 3 - 24
13 hours after 300mg/kg APAP. However neutrophils were not apparent in livers of mice treated with a
14 non-toxic dose of APAP (175mg/kg) but significant accumulation occurred when this dose was co-
15 administered with a non-injurious dose of LPS. Two hours after treatment with LPS (0 hours after
16 APAP), serum TNF- α levels were large and rapidly decreased thereafter, confirming that the
17 inflammatory response observed in this model was due to LPS [115]. These observations support the
18 hypothesis that inflammation induced by LPS and associated elevated levels of TNF- α increase
19 sensitivity to AILI.
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30 Fasted transgenic C57BL6 mice lacking anti-inflammatory mediators such as IL-10, IL-4, IL-6, MIF
31 and IL13 dosed with APAP (200-300mg/kg, i.p) displayed increased susceptibility to AILI [109,116-
32 118]. Neutrophils were found to accumulate to a greater extent in IL13 KO mice than wild-type
33 treated with APAP with the level of TNF- α , IL-6 and IFN- γ increased at 4 hours in WT and IL-13 KO
34 APAP-treated animals compared to saline-treated controls [118]. These data suggest a
35 hepatoprotective role for anti-inflammatory mediators in these models and that the balance
36 between the Th1 and Th2 response is an important determinant for AILI. Susceptibility to AILI was
37 compared between two mouse strains, C57BL6 (more susceptible to AILI and which develop
38 predominantly a Th1 response) and BALB/c (less susceptible to AILI and which develop a Th2
39 response). 24hr after exposure to APAP C57BL6 mice produce high levels of TNF- α (pro-
40 inflammatory) whereas levels in BALB/c mice show no difference. On the other hand IL-6 (anti-
41 inflammatory) levels were higher in BALB/c than C57BL6 24 hours after administration. These data
42 support the hypothesis that AILI is associated with a Th1-dominant response in Th1/Th2 cytokine
43 balance and TNF- α may play a role in toxicity [117].
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56 However, the role of TNF- α remains controversial with contradictory reports suggesting that
57 TNF α is involved in pathogenesis of DILI [105-106]; is not involved in toxicity [104-119] or it has a
58 role in defence against toxicity and repair [120-121]. Data suggests that like most cytokines the
59 effects of TNF α in liver toxicity are pleiotropic with other cytokine networks being involved and it is
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the interplay between these different cytokine cascades that determine the nature of the inflammatory response associated with toxicity. IL1 α [106] and INF γ [108] have been shown to play a pathogenic role in APAP-induced hepatotoxicity whilst IL-6 [117] and IL10 [116] play a protective role. Furthermore, the understanding of the regulatory mechanisms in the context of DILI that control the potential for DAMPS or cytokines to activate immune cells and initiate an inflammatory response require further investigation. For example, recent evidence suggests that the caspase-dependent oxidation of HMGB1 is critical for inhibiting its immune stimulatory potential following apoptosis [16].

As well as APAP, other model hepatotoxins have been used to investigate the role inflammation plays during drug-induced liver injury (Table 3). Given the complexity of interplay between chemically mediated toxicity and inflammation, and the differences observed between strains of mice, species and *in vivo* model pre-treatment further investigation is required to define the precise role a cytokine has in response to the injury or as part of the inflammatory response. Once this has been defined then the value of the cytokine as a biomarker can be assessed.

Table 3: Inflammation in other models of DILI

| DILI Model | Inflammatory factors investigated | References |
|---------------------|-----------------------------------|------------|
| LPS | Neutrophils, TNF- α , KCs | [122-124] |
| EtOH | Kupffer cells, TNF- α | [125-128] |
| Halothane | Macrophage inhibitory factor | [116] |
| Cocaine + LPS | Kupffer cells, nitric oxide | [129, 130] |
| Galactosamine + LPS | TNF- α , TNF-p55 receptor | [131, 132] |

Cytokine expression in clinical studies of acetaminophen-toxicity

As has been discussed many studies have investigated the role of cytokines in animal models. Some of these cytokines have been reported to have hepatoprotective effects in animal models and several studies have been performed to investigate cytokine expression patterns in serum samples from different patient cohorts. In a study of 111 patients (90 female; mean age 13.6 years) plasma concentrations of IL6, IL8, IL10 and Monocyte chemoattractant protein 1 (MCP1) were analysed by enzyme-linked immunosorbent assay. Patients were stratified by toxicity severity as defined by the maximal values of hepatic transaminase elevation. Levels of IL6, IL8 and MCP1 were elevated in patients with elevated serum alanine aminotransferase and MCP1 expression had the strongest association with toxicity [133]. Other studies have found associations between elevated

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3 expression levels of IL8 [134, 135]; IL6 [136] and acetaminophen-induced hepatotoxicity. While the
4 results from these studies indicate that inflammation and cytokines are associated with the
5 progression of late stage toxicity interpretation of the data is often limited by the absence of pre-
6 dose data, a lack of samples from early in the process of drug-induced injury and the small number
7 of samples usually obtained in the clinic.
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14 Several other studies have investigated the expression of various cytokines associated with
15 various forms of acute liver failure (ALF). In a mixed cohort of patients with ALF and acutely
16 decompensated cirrhosis it was found that not only was the degree of systemic inflammation, as
17 measured by IL6 and TNF α , important for determining outcome but that the magnitude of the anti-
18 inflammatory response was also important. In the whole cohort, IL10 (regarded as an anti-
19 inflammatory cytokine) levels were found to predict outcome as accurately as IL6 [137]. However
20 these patients are experiencing acute liver failure and while expression of the cytokine is linked with
21 disease progression the patient cohorts are characterised by end stage severe liver failure and it is
22 therefore difficult to relate cytokine expression patterns with the earliest stages of injury when
23 intervention is required to prevent toxicity from developing further.
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34 Recently a couple of studies have been reported which attempted to address the issues
35 surrounding patient sampling by treating volunteers with a bolus of acetaminophen under
36 controlled conditions. This has allowed the determination of a pre-acetaminophen treatment
37 baseline for parameters and accurate timing of sampling post-acetaminophen dose. DNA
38 microarrays and serum metabolomic methods were employed to characterise changes in the
39 transcriptome and metabolome in serial peripheral blood (PB) samples obtained from six healthy
40 adults treated with a 4-g bolus of acetaminophen and from three receiving placebo. Treatment did
41 not cause liver toxicity as assessed by traditional liver chemistries. It was found that a single 4-g
42 bolus of acetaminophen produced a transcriptomic signature in PB cells characterised by down
43 regulation of oxidative phosphorylation genes accompanied by increased serum lactate. Similar gene
44 expression changes were observed in rats and several patients after consuming hepatotoxic doses of
45 acetaminophen [138]. While this study is exciting as it begins to allow an insight into the progression
46 of acetaminophen induced liver injury in a controlled setting, it also highlights the challenges faced
47 in identifying novel biomarkers for DILI that can be applied in the clinic. The changes reported in this
48 paper occurred 48 hours after the acetaminophen was administered to volunteers and are therefore
49 likely to be downstream of liver injury. The authors also recognise that the mechanism by which liver
50 toxicity affects peripheral blood cells is unclear, are the changes observed direct or indirect effects?
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In a separate study it was shown that urine metabolite profiles from healthy adults each receiving 4g of acetaminophen per day for 7 days profiles obtained before start of treatment were not sufficient to distinguish which of the subjects would develop mild liver injury. However profiles obtained shortly after the start of treatment but prior to ALT elevation, could distinguish responders from non-responders [139]. This data suggests that an early-intervention pharmaco-metabolomics approach could be used to identify potential toxicities early, however much more work is required to validate this approach. However in both these studies it is interesting to note that few, if any, of the changes described were associated with cytokines. Analysis of liver chemistries showed that liver toxicity had not been caused by the dose of paracetamol used, therefore it indicates supports the hypothesis that inflammation and cytokines are involved only after overt liver damage has occurred.

Immunological involvement in idiosyncratic DILI – the example of halothane

Lessons from animal studies have identified the potential role of the innate immune response in dictating the overall response during APAP hepatotoxicity; however, this stills needs to be proven in man. While the role of immune cells, and by extension cytokines, remains unclear other approaches are been taken to identify biomarkers. Some of the characteristics of idiosyncratic DILI reactions appear to have an immune component and as many autoimmune functions appear to be mediated by TH17 cells, it was hypothesised that IL17 may play a role in iDILI [140]. Expression patterns of cytokines were examined in the serum of 39 patients with acute liver failure (ALF) due to IDILI and compared the values with those from 21 patients with acetaminophen-induced ALF and 10 patients with viral hepatitis-induced ALF. IL-17 levels were found to be elevated in a similar number of patients IDILI and acetaminophen-induced ALF and occasionally in patients with viral hepatitis. There was overlap in the level of expression of IL-21 in patients with iDILI and ALLI. Auto-antibodies were more frequent in patients in the IDILI group but were absent in most patients. These data provide a picture of the cytokine/chemokine profile in patients with various types of ALF. The pattern varies from patient to patient and not specifically by etiology. This suggests that different underlying disease mechanisms may be at play in different individuals, even among those demonstrating injury from the same drug. Since cytokines may originate from more than one type of cell, interpretation of results of cytokine assays remains difficult in complex disease settings.

However it is certainly possible that an adaptive immune response may play a role in cases of human DILI. Certain drug reactions have clinical hallmarks of an immunological mechanism, the liver alone may be involved or liver injury may be part of a more complex hypersensitivity syndrome.

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3 Unlike with APAP, where the initiation of hepatotoxicity is dependent upon the intrinsic chemistry of
4 the molecule, these so called idiosyncratic hepatotoxicity reactions represent a function of an
5 individual patient's biology (Table 4).
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9 The inhalation anaesthetic halothane is the best-studied drug causing an immunoallergic
10 response in a subset of patients. The response ranges from asymptomatic rises in transaminases to
11 fulminant hepatitis, which is rare but serious. Most of the patients recorded in the literature with
12 immunoallergic hepatitis had more than one exposure [141]. Antibodies have been detected in such
13 patients that recognise autoantigens and neoantigens created by trifluoroacetylation (TFA) of
14 hepatic proteins. Pre-incubation of halothane pre-treated rabbit hepatocytes with sera of patients
15 with halothane-induced fulminant hepatitis increased susceptibility to cytotoxicity of lymphocytes *in*
16 *vitro* [142]. It is likely drug specific T cells play a role in the pathogenesis of hepatocyte injury but
17 direct evidence is lacking. Halothane is metabolised by CYP450 2E1, like APAP, but forms a
18 chemically reactive acyl halide and bioactivation to the acyl halide is substantial as it is the only
19 metabolic route available. It is hypothesised that the formation of the acyl halide triggers an immune
20 response and that mild and severe immune responses are likely to have this common initiation
21 mechanism. Evidence for this concept of an immune trigger is both direct and indirect, with drug
22 specific antibodies indicative that the drug has initiated an immune response in affected patients
23 and newer metabolically inert inhaled anaesthetics being rarely associated with hepatotoxicity.
24 Target proteins modified by the acyl halide have been identified with the principal chemical
25 modification trifluoroacetylation of lysine residue [143-146]. Chemical modification of protein(s) is
26 associated with the immune response observed with halothane hepatitis, however, there is no
27 conclusive evidence that the antibodies mediate liver injury. Antidrug antibodies have also been
28 observed with idiosyncratic drug reactions to Tienilic acid [147] and dihydralazine [148] but there is
29 no unequivocal evidence that DILI such as that caused by halothane is immune mediated. Most of
30 the information available is compatible with the hapten hypothesis where the drug undergoes
31 bioactivation in hepatocytes leading to drug-protein conjugate formation in the liver. The current
32 theory suggests that the occurrence of idiosyncratic drug hypersensitivity in man represents
33 interplay between a number of factors, which include the chemistry of the drug, drug metabolism
34 and the immune system of the patient (Table 4). Whether cytokines may be useful biomarkers in
35 such idiosyncratic cases is currently unknown as the data supporting immune involvement is
36 equivocal and the mechanistic details are unknown. However, if the hapten hypothesis is correct
37 what is clear is that the inflammatory response induced by chemical insult such as APAP overdose
38 compared to that of idiosyncratic DILI may be very different.
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Table 4: Idiosyncratic drug reactions with evidence of immune involvement (amended from [149])

| Drug Class | Drug Name | Is there evidence to support immune involvement? |
|-------------------------|---------------------------|--|
| Anaesthetic | Halothane | Yes |
| Analgesic | Diclofenac | Yes/No |
| | Sulindac | Yes |
| Antibiotic | Amoxicillin | Yes |
| | Erythromycins | Yes |
| | Minocycline | Yes |
| | Nitrofluantoin | Yes |
| | Rifampicin | Yes/No |
| Anti-convulsant | Phenytoin | Yes |
| | Phenothiazines | Yes |
| Anti-epileptic | Carbamazepine | Yes/No |
| Antihypertensive | Dihydralazine | Yes |
| | Methyldopa | Yes |
| | Tienilic acid | Yes |
| Anti-thyroid | Propylthiouracil | Yes |
| Depression | Tricyclic antidepressants | Yes |
| Gout | Allopurinol | Yes |
| Hypertension | ACE inhibitors | Yes |

Conclusions and perspectives

An analysis of the role of inflammation in human liver disease and in the *in vivo* models described offer insights into the potential role of inflammation in human DILI. Given the role of cytokines in modulating the inflammatory response changes in patterns of cytokine expression certainly show promise as potential biomarkers of DILI. However, the *context* of cytokine expression will determine their utility as biomarkers. This context will be determined by understanding the processes within DILI and the associated inflammatory cascade in which the cytokine has a role (Figure 1). One key question is whether the inflammatory response precedes the injury or is a consequence of the injury (Figure 2). Also, inflammation is associated with a lot of confounding factors experienced in the clinic (as previously described) therefore much more data is required in order to support the context of a given cytokines' qualification for use as a biomarker and the proposed application of that biomarker. Clearly prior to selecting a given cytokine as a biomarker it

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3 is important to accurately phenotype DILI and to have a thorough understanding of the processes
4 and mechanisms by which injury occurs.
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9 As discussed the balance of pro and anti-inflammatory cytokines is thought to play a
10 significant role in dictating the overall balance of hepatic injury exacerbation or regeneration, in
11 particular for DILI characterised as idiosyncratic in nature (Figure 2). The majority of animal models
12 of DILI are exposed to profound, often lethal levels of chemical stress with changes in the expression
13 of cytokines such as TNF α , HMGB-1, IL6, IL10 and IL1 β [16, 18-20]. Therefore animal model data may
14 be misrepresentative of the majority of cases of liver injury observed clinically. The promotion of
15 inflammation may be the most significant consequence of chemical stress in the exacerbation of
16 DILI, in which case, cytokines remain an attractive option as biomarkers of DILI to inform the
17 mechanism of pathology, sensitively screen for hepatic injury and predict at risk patients.
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26 Even when a cytokine has been shown to be a candidate biomarker the benefit from use of a
27 novel biomarker in terms of safety efficacy and or cost must be clearly established. Biomarkers must
28 be sensitive and specific and this requires accurate definition of the context for which a biomarker
29 should be qualified [150]. Context and qualification for new biomarkers are assessed relative to
30 current biomarkers. If the sensitivity and specificity of current biomarkers are not perfect relative to
31 a specific end point, the context and qualification of new biomarkers may not be accurately
32 established. This is a particularly difficult problem if current biomarkers have pseudo-quantitative,
33 qualitative, or subjective metrics associated with them. Even the data available for the gold standard
34 of DILI (ALT) is continually reviewed to ensure that the context of its use is as accurate as possible
35 [151].
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45 Much remains to be understood about the role of inflammation in DILI and cytokines in
46 controlling this inflammatory response. Cytokines are interesting candidates for novel biomarkers as
47 they are relatively accessible (by blood sampling) and quantifiable. However without fully
48 understanding the role of inflammation in DILI they may be limited in their use to being diagnostic of
49 the type of injury that has occurred and maybe prognostic of outcome (recovery from DILI, cirrhosis,
50 acute liver failure). Intracellular components released by damaged hepatocytes, although
51 inaccessible and currently difficult to quantify may be better biomarkers for the prognosis of
52 whether an injury will occur (Figure 1). However, further investigation is required to understand the
53 impact of chemical stress on cellular physiology and the release of intracellular components in
54 response to injury as well as the development of assays sensitive enough to accurately detect them.
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Figure Legends

Figure 1 Paracetamol-induced hepatotoxicity: a multistep/multicellular process

The multi-step process and multi-cell type involvement during acetaminophen-induced hepatotoxicity. Acetaminophen (APAP) hepatotoxicity is dependent upon the metabolic activation by CYP450 to the reactive metabolite NAPQI. Following saturation of the phase II conjugation detoxification pathways, NAPQI becomes covalently bonded to hepatic protein (in particular mitochondrial protein) which leads to cell death. Cell death can proceed through either apoptosis or necrosis depending upon the bio-energetic status of the cell. So called DAMPs (e.g HMGB-1) are released from necrotic cells which hold the potential to activate the innate immune response. Evidence from animal models suggests that the overall extent of hepatic damage is dictated by the balance between the production of pro and anti-inflammatory cytokines. The dependency upon the multiple steps has been elucidated experimentally in vitro and in vivo from CYP450 dependence (inhibitor; ABT), glutathione level (NAC; N-acetyl-cysteine), mitochondrial permeability transition (CsA; cyclosporine), and cellular ATP status (dietary restriction) to caspase activation (inhibitor; Z-VAD.fmk)

Figure 2 Inflammatory etiology of acute liver failure

Acute liver failure stimulated by a variety of inducing agents leading to the development of the pathology often share common pathways through the activation of pro-inflammatory pathways. Initial necrotic cell death from reactive metabolites, drug accumulation, diet, alcohol or viruses can lead to the release of DAMPs which in turn activate the primary and secondary inflammatory cascades. Underlying hepatitis can influence an individual's susceptibility to hepatotoxicity. Understanding the mechanisms involved from the study of liver diseases or the particular serum cytokine finger print when applied to DILI may help to predict susceptible individuals or patients likely to develop severe hepatotoxicity

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The Potential of Cytokines as Safety Biomarkers for Drug-Induced Liver Injury

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Abstract

Drug-induced liver injury (DILI) is an event that has a detrimental impact on drug development and patient safety, therefore the identification of novel biomarkers that are not only sensitive and specific to the liver would have great benefit. Inflammation is known to be associated with human cases of DILI and given the role of cytokines in modulating the inflammatory response changes in cytokine expression patterns certainly show promise as potential biomarkers of DILI. Cytokines are interesting candidates for novel biomarkers as they are relatively accessible (by blood sampling) and accurately quantifiable. In particular, recent interest has developed in mechanism, rather than tissue, specific biomarkers. However, without fully understanding the role of inflammation in DILI, and the role of cytokines in modulating the inflammatory response, they may be limited in their use, being either diagnostic of the type of injury that has occurred and/or prognostic of outcome (recovery from DILI, cirrhosis, acute liver failure). Intracellular components released by damaged hepatocytes, although inaccessible and currently difficult to quantify, may be better biomarkers for the prognosis of severity of injury. In both cases there is a pressing need for the development and validation of assays sensitive enough and with a sufficient dynamic range to detect changes upon drug treatment. Although promising candidates are appearing in the literature much remains to be done to understand the role of inflammation in DILI and the role that a given cytokine has in the inflammatory cascade associated with DILI before cytokines are viewed as biomarkers for DILI.

Key words

Cytokine, inflammation, pharmaceutical, biomarker, drug-induced liver injury

Introduction

Drug Induced Liver Injury (DILI) is an adverse event that leads to regulatory action on drugs culminating in serious instances of withdrawal from the market. At a regulatory level hepatotoxicity is the single most frequent reason for removing approved medications from the market. Between 1975 and 1999, 548 new drugs were approved by the FDA of which 10 received a 'black box warning' for potential hepatotoxicity and an additional four were withdrawn from the market [1]. The population incidence of drug-induced liver injury is unknown as most events are often inaccurately classified [2-4]. Yet DILI is the most frequent cause of acute liver failure among those under consideration of liver transplantation in US [5], in the UK alone paracetamol (acetaminophen, APAP) overdose accounts for 200-500 deaths and 20-40 liver transplants annually [6]. In drug development animal studies detect approximately half of compounds exhibiting hepatotoxicity in man [7], whereas *in vitro* human hepatocyte testing detects 50-60% of drugs that can cause severe liver injury in man, including some not detected by animal testing [8]. So although current testing methodologies are capable of detecting some hepatotoxins in drug development there are no currently available preclinical tests that detect the potential for serious or idiosyncratic human hepatotoxicity with combined high sensitivity and specificity.

If drugs capable of causing serious DILI at a very low frequency are identified, there is a need for reliable biomarkers that could be used to predict which individuals are susceptible to develop DILI from that drug either before drug exposure, or at a very early time after drug treatment has begun (e.g., assays of substances in blood or urine). Ideally, these biomarkers would discriminate between individuals susceptible to, those capable of adapting to and those that can tolerate DILI. Upon their validation, such biomarkers could facilitate approval of drugs that would otherwise not be possible, by excluding identified susceptible people who should never be exposed to that particular drug [9].

To be able to identify novel biomarkers for evaluation of new chemical entities or determination of individual patient susceptibility requires a thorough understanding of the mechanisms involved in DILI. Although the pathogenesis of DILI is not fully understood, studies of mechanisms in animal models can be used to split DILI into a two stage process. Stage I often involves either excessive drug accumulation, biotransformation and metabolic activation, which can be followed by covalent binding and transport when bioinactivation processes become saturated or immobilized resulting in chemical stress which in turn leads to a cellular effect e.g., mitochondrial dysfunction (Figure 1). The identification of biomarkers at this stage is difficult unless a 'drug

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2 related-metabolite' or a modified protein is secreted into the blood stream and/or urine at levels
3 that can be detected. Often the earliest changes observed in safety screening are histopathological
4 and therefore not practical for use as a biomarker in humans. Stage II represents subsequent
5 processes of adaptation or failure of response to modification of essential cellular processes [10]
6 which may be partially mediated by liver nonparenchymal cells, Kupffer cells and/or infiltrated
7 macrophages [11-13]. The changes within the liver at this stage of DILI are more amenable to
8 biomarker discovery. Injured hepatocytes release factors that are chemoattractant and subsequently
9 activate non-parenchymal cells potentially compounding cellular damage by releasing cytotoxic
10 mediators. Although the mediators for progression of DILI have not yet been absolutely defined,
11 recent evidence supports the hypothesis that the types of cytokine produced in response to the
12 initial insult influence the severity of the toxicity [14]. Can we use human liver disease and animal
13 models, where inflammation is known to play a role to better understand the role of inflammation in
14 DILI and in so doing identify novel biomarkers for DILI or identify potential strategies for therapeutic
15 intervention.
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24 **Clinical features of DILI and current assessment**

25 Due to complexity within the structure and the function of the liver, clinical symptoms of
26 drug-induced hepatotoxicity may take many forms. DILI may mimic various forms of naturally
27 occurring liver disease, reviewed previously [15]. The assessment of DILI is primarily based on the
28 relatively non-invasive quantification of blood protein biomarkers and hallmarks of hepatic
29 biochemistry. The clinical manifestations of DILI range from mild asymptomatic biochemical changes
30 to fulminant hepatic failure. However, the most useful form of differentiation of hepatic pathology is
31 between cholestatic or hepatocellular injury [15] which is based on the differential quantification of
32 blood hepatic enzymes (Table 1).
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38 Hepatic blood enzymes are roughly grouped in to hepatocellular markers, such as serum
39 alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and the cholestatic markers
40 alkaline phosphatase (ALP), and γ -glutamyltransferase (GGT). These enzymes are most often elevated
41 in patients with liver disease and may reflect liver injury. However, they are present in tissues
42 throughout the body and often physicians are faced with the problem of a wide differential diagnosis
43 with low specificity. Currently there are no pathognomonic indicators of DILI; even liver biopsy is not
44 diagnostic, while confounding factors such as viral hepatitis or congestive heart failure may not be
45 recognized in the setting of hepatic illness. In certain patient groups, such as diabetic or obese
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2 persons, for example, liver test abnormalities are common, and other medical conditions such as
3 coronary artery disease and renal failure may confound clear identification of affected patients.
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6 The sensitivity of these tests is questionable. In clinical trials mild elevations of ALT or AST
7 are used as markers of DILI. However, the thresholds used are not consistent between trials in
8 defining DILI: variously placed at twice, thrice, five times or even ten times the upper limit of the
9 normal reference range, but are typically below 40 IU/L. ALT is thought to be a more specific
10 indicator than AST, but normal values vary greatly between laboratories [16]. Despite the association
11 between greatly elevated ALT levels and hepatocellular diseases, the absolute height of the ALT
12 elevation does not correlate with the extent of liver cell damage [17]. Transient serum enzyme
13 activity elevations, however high, are common and can be confusing. The physician is never sure
14 whether they would translate into potentially serious clinical symptoms of hepatic illness.
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21 The use of the current normal range may lead to an underestimated prevalence of liver
22 disease [18]. Because the enzyme concentration in a population forms a continuous distribution, the
23 cut-off concentration that discriminates between healthy and diseased livers is not clearly defined
24 [19]. Indeed several studies have recently questioned whether previously established values to
25 define normal ALT range are accurate and have suggested that the upper limit of normal should be
26 revised [20]. Various scoring tools have been developed to assess causality (RUCAM) usually
27 involving complex calculation of probability based on ALT, ALP, GGT ratios weighed against time of
28 onset, age, alcohol use, viral hepatitis infections, biliary tract disease, or hepatic ischemia. The
29 validation, accuracy and scientific basis of these tools has been problematic. Powerful computing
30 tools and sophisticated Bayesian algorithms [21] have been used but these are not readily available
31 for the clinician at the bedside.
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38 Bilirubin cannot be reliably used as a marker of early liver injury. Once again the threshold is
39 ill-defined and once arbitrarily set can be a late marker of liver injury. Many disease processes and
40 inherited disorders such as Gilbert's syndrome may lead to elevated levels of bilirubin not related to
41 DILI and may falsely raise alarm bells to the treating physician. The appearance of jaundice is often a
42 late marker of disease. Another commonly used test, the blood prothrombin time (or its derivative
43 Internationalized Ratio, INR) may be useful as a liver function test (of protein synthesis), however,
44 individual laboratories have to determine their own reference values. In paracetamol overdose the
45 INR can be used prognostically especially on day 2 or 3 to determine deterioration or improvement.
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47 However, this too is a late indicator of liver dysfunction and initially may not predict which patients
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are likely to deteriorate. Arterial blood lactate measurement rapidly and accurately identifies patients who will die from paracetamol induced acute liver failure. Its use could improve the speed and accuracy of selection of appropriate candidates for transplantation.

Consequently there is no specific non-invasive diagnosis, treatment or prevention of DILI except for the early withdrawal of a drug treatment to the individual in the case of suspected DILI. Currently, the monitoring of the parameters described in Table 1 represents the best indication of hepatic injury. Therefore newer, rapid and reflective non-invasive biomarkers are required that are not only sensitive for hepatic injury but also provide insight into the mechanism of pathology. There are a number of potential biomarkers reflective of hepatocellular damage which may offer superiority over ALT determination [22]. However, there are a limited number of candidates reflective of other pathologies observed during DILI, namely hepatitis, steatosis, fibrosis and cholestasis. Therefore, a comprehensive understanding of the mechanisms that lead to the development of these conditions is required to reveal potential biomarkers specific for established liver pathologies.

Table 1: Standard panel of currently used blood clinical chemistry parameters to assess hepatic function and injury. Taken from [23].

| Biomarker | Injury type | Normal reference range |
|--------------------------------------|------------------|----------------------------------|
| Alanine aminotransferase (ALT) | Hepatocellular | 5-40 IU/L |
| Aspartate aminotransferase (AST) | Hepatocellular | 10-40 IU/L |
| Alkaline phosphatase (AP) | Cholestatic | 30-120 IU/L |
| Gamma glutamyl-transpeptidase (GGT) | Cholestatic | 0-51 IU/L |
| Total bilirubin | Hepatic function | 2-14 $\mu\text{mol/L}$ |
| Direct (conjugated) bilirubin | Hepatic function | 0-4 $\mu\text{mol/L}$ |
| Pro-thrombin time (coagulation test) | Hepatic function | Local laboratory reference value |

Biomarkers of DILI

Biomarkers are defined as an objectively measured change in either DNA, protein or metabolite levels that is reflective of a normal biological process, pathological condition or correlates with the pharmacological action of a therapeutic agent [23, 24]. The ideal DILI biomarker would have the following characteristics:

- Reflective of the actual hepatic injury/repair status
- Rapidly assayed and can be assayed in any laboratory
- Non-invasive, i.e. present in the blood or urine compartment
- Transferable across *in vitro*, *in vivo* and clinical systems to further inform and accelerate drug safety screening
- Able to precede the onset of massive hepatic injury – secreted markers rather than leakage
- Of a low base-line variability in control samples and normal population
- Accurately identifiable and quantifiable with a suitable dynamic range to detect upon drug treatment

The current clinical standard for assessing inflammation by a blood borne biomarker is through the quantification of C-reactive protein (CRP). CRP is an acute-phase protein which is produced rapidly in response to inflammation and infection. The normal physiological role of CRP is to activate the complement system by C1q by binding phosphocholine expressed on the surface of dead / dying cell (and bacteria) which enhances phagocytosis by macrophages. CRP is synthesised in the liver, therefore CRP holds little utility as a marker of inflammation when hepatic dysfunction or DILI is suspected [25].

Taking the characteristics above in to account, cytokines as potential novel biomarkers of DILI, in particular idiosyncratic DILI, represent promising candidates. With underlying inflammation thought to be a contributing factor for the onset of idiosyncratic DILI [26-29], the quantification of cytokines as potential predictive markers of patient susceptibility holds promise. However, the large variation observed in base-line levels in normal populations renders the establishment of reference values to assay meaningful elevations difficult. There are many established and robust commercially available ELISA assays utilising non-invasive biofluids for the accurate absolute quantification of cytokines. Ease of assay use, the presence in a non-invasive biofluid and the species cross reactivity of antibodies used in ELISAs also aids the translation between pre-clinical and clinical studies. These factors represent the main advantage of cytokines as biomarkers of DILI [30]. In healthy volunteers and control pre-clinical test species with tightly controlled diets and environments cytokine

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2 elevations often exhibit a large dynamic range upon drug treatment. However, it is important to
3 note that added sensitivity and dynamic range may give rise to an increase in false positives, which
4 in turn may lead to the failure to progress an otherwise safe candidate in development or the
5 withdrawal of potential efficacious drug treatment.
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10 When assessing novel candidates as biomarkers, it is important to have a comprehensive
11 understanding of biomarker kinetics and distribution. The active secretion of cytokines from cells
12 present in the liver and at distant sites indicates that blood elevations may occur prior to cell death
13 or organ dysfunction in response to mild, non-toxic cell stress. Moreover, given the short half life of
14 cytokines, they return to background levels rapidly and therefore may also represent markers of
15 reversible hepatic damage, such as that shown recently for High Mobility Group Box-1 protein
16 (HMGB-1) in a mouse model of acetaminophen hepatotoxicity [31]. Focussing upon the kinetics of
17 blood biomarker appearance and a particular signature pattern of cytokine expression may give a
18 real insight into hepatic function and toxicity state. However, caution should be taken in the
19 interpretation of multiple biomarker kinetics because many of these biomarkers are not organ
20 specific. Novel, possibly non-specific, biomarkers of damage should always be assessed alongside the
21 gold standard biomarkers for that organ, e.g. ALT/AST for hepatic damage. The correlation between
22 pro-inflammatory cytokine level changes in blood (increase/decrease) with the extent of liver
23 damage or established biomarkers is not fully known and warrants further investigation.
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31 **The Role of inflammatory Cells and Cytokines in Liver Disease**

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33 Given that cytokines have several of the features required for novel, mechanism biomarkers
34 what data is available in terms of human disease and liver injury for their use as a biomarker in drug
35 safety. Human liver disease takes many forms and can result from viral hepatitis [31], obesity (fatty
36 liver disease; [32]), alcohol abuse (fatty liver disease, alcoholic hepatitis, cirrhosis [33]), genetics,
37 autoimmune disorders (primary biliary cirrhosis, primary sclerosing cholangitis and autoimmune
38 hepatitis [34]). As the inflammatory component has been best described in the cases of viral hepatitis
39 and alcoholic liver disease we focus on the common themes in inflammation associated with these
40 two types of liver injury.
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45 **Viral Hepatitis**

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47 At all stages of viral hepatitis infection chemokines are key regulators of immunity and
48 inflammation functioning within cytokine cascades that regulate the immune response to the virus.
49 However in chronic infection their persistent expression can drive chronic inflammation leading to
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2 liver injury and cirrhosis [36]. The Hepatitis B Virus (HBV) itself is non-cytopathic but immune
3 mediated liver damage often occurs in patients with both acute and chronic HBV infection. Such
4 damage has been attributed to the killing of infected hepatocytes by virus specific toxic CD8+ T cells
5 [37, 38]. There is increasing evidence to suggest that a non-HBV-specific inflammatory infiltration
6 into the liver is likely responsible for hepatic pathology in patients with chronic hepatitis B [39, 40].
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11 Upon infection with Hepatitis C Virus (HCV) hepatocytes secrete interferon (IFN) and down
12 regulate RNA translation as a response to infection before innate immune cells, including
13 macrophages, dendritic cells (DC), natural killer cells and natural killer T cells are activated to amplify
14 secretion of type-1 IFNs and IFN-response genes. Activation of Toll-like receptors, triggers
15 chemokine secretion which amplifies leukocyte recruitment [41, 42]. The activation of innate
16 immunity by the uptake and processing of viral antigen by DCs is followed by the migration of DCs to
17 the lymph nodes where they present viral antigen to naive T cells leading to their activation and the
18 development of an adaptive immune response [43]. Two types of DCs have been characterised in
19 the response to HCV infection of hepatocytes, myeloid dendritic cells (mDCs) and plasmacytoid
20 dendritic cells (pDCs). pDCs secrete type-1 IFN, prime T helper 1 (Th1) responses and activate
21 cascades of chemokine secretion in HCV infection [44]. mDCs prime Th1 responses via interleukin 12
22 but produce little type-1 IFN [44, 45]. In the early phases of viral infection chemokines particularly
23 CXCL8, CXCL16, CCL2 and CCL3 promote recruitment of innate immune cells to the liver, including
24 DCs which then initiate the adaptive immune response and it is likely that similar events shape the
25 response to HCV infection [46-47].
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34 TH1 immune responses dominate in the HCV-infected liver (Shields et al., 1999) and
35 intrahepatic T cells express chemokine receptors associated with Th1 responses eg., CXCR3 [49].
36 Hepatic CXCR3 ligands are increased in many liver diseases and this suggests that they play a role in
37 effector cell recruitment to the inflamed liver [50]. Expression of CXCR3 is closely linked to Th1
38 function and its ligands CXCL9, CXCL10 and CXCL11 are induced by the TH1 cytokines IFN γ and TNF α
39 [51, 52]. In contrast to HCV, HBV infections are characterised by increased numbers of TH17 cells
40 producing a cocktail of cytokines of which IL17A is characterised as a major effector cytokine [53].
41 IL17A can mobilise, recruit and activate neutrophils leading to massive tissue inflammation and
42 promote the progression of autoimmune disease [54]. Peripheral and intrahepatic TH17 cells are
43 preferentially increased in patients with chronic HBV infections, which may activate mDCs and
44 monocytes to release inflammatory cytokines during chronic HBV infection [55].
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Viral clearance is associated with a vigorous CD4+ and CD8+ T cell response again with Th1 responses dominating. Regulatory T cells (Tregs) have been found in the HCV-infected liver and appear to have a dualistic effect, they suppress HCV-specific CD8+ T cells and promote viral replication however they also appear to suppress collateral inflammatory damage to reduce liver injury in chronic infection [56, 57]. Successful antiviral therapy is associated with an increase in circulating CXCR3+CD8+ T cells and a reduction in CXCL10 and CXCL9 levels in blood [52, 58, 59]. Interestingly the vasculitic lesions of cryoglobulinemia in nerves and skin are characterised by upregulation of CCL3, CCL4 and CXCL10 and infiltration by CXCR3+ Th1 cells and CCR5+ monocytes. Thus the same pro-inflammatory chemokines are implicated in vasculitic complications of globuliemia and hepatitis [60].

Alcohol Liver Disease

Factors such as gender, age, nationality, weight and health can affect how a person's liver metabolizes alcohol. When the liver has too much alcohol to handle, normal liver function may be interrupted leading to a chemical imbalance. If the liver is required to detoxify alcohol continuously, liver cells may be destroyed or altered resulting in fat deposits (fatty liver) and more seriously, either inflammation (alcoholic hepatitis) and/or permanent scarring (cirrhosis). Liver cancer can also result from alcohol induced liver disease. It is increasingly evident that chronic inflammation represents the driving force in the evolution of alcohol-induced liver injury to steato-hepatitis and cirrhosis. Accordingly, the hepatic expression of pro-inflammatory cytokines precedes the development of histologic signs of necro-inflammation in alcohol fed rats [61], while steato-hepatitis is associated with leucocyte focal infiltration and elevated circulating levels cytokines/chemokines such as TNF α , IL1, IL6, IL8/CINC and macrophage chemotactic protein-1 (MCP1) [62-65].

Innate and adaptive immunity in alcohol liver disease

The interaction between alcohol and hepatic inflammation is complex. Early studies proposed the involvement of innate immunity with the increased translocation of gut-derived endotoxins to the portal circulation being recognised by Kupffer cells via the toll-like receptor-4 (TLR4 [63, 66]). Ethanol itself has been found to enhance the capacity of Kupffer cells to respond to pro-inflammatory stimuli via the up regulation of Toll-like receptors [67] and the production of pro-inflammatory cytokines and chemokines by modulation of signalling pathways such as nuclear transcription factor κ B (NF κ B), early growth response-1 (ERG1) as well as JNK, ERK and p38 protein kinases [65, 68-70].

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Histologic examination of liver biopsies from patients with alcoholic hepatitis reveals the presence of CD4+ and CD8+ T-lymphocytes [71]. The influence of adaptive of immunity in the response to alcohol is supported by the predominance of Th1 cytokines (high TNF α , INF γ , IL1) in peripheral blood T-cells isolated from active drinkers with or without ALD [72]. Activated liver-infiltrating IL17-producing T Helper (TH17) cells are also responsible for neutrophil recruitment into the liver [73]. Furthermore serum IL17 levels are increased and serve as a marker of the severity of acute hepatic injury [74]. Evidence is accumulating to suggest that TH17 lymphocytes have an important role in driving inflammation during the development of other chronic inflammatory and autoimmune diseases including inflammatory bowel disease, psoriasis rheumatoid arthritis and multiple sclerosis [75] as well as viral hepatitis as has been discussed.

Alcohol may also have an effect on the immune system by effects on osteopontin, leptin and adiponectin. Osteopontin is a cytokine that enhances the pro-inflammatory Th1 response and the livers of alcohol fed rats have been found to have an associated increase in osteopontin levels [76] while patients with alcoholic hepatitis have higher levels of osteopontin mRNA in their livers compared to patients with fatty liver only [77]. Leptin has been shown to have a pro-inflammatory function while adiponectin counters pro-inflammatory cytokine production [78]. Alcohol appears to have opposite effects on these adipokines, patients with ALD have increased serum leptin [79] whereas chronic ethanol feeding decreases adiponectin secretion in the early phase of alcohol injury in rodents [80, 81].

Signal transduction pathways

In response to alcohol several different signal transduction pathways are activated. Transcription factors regulating fatty acid metabolism including sterol regulatory binding protein (SREBP) and peroxisomal proliferating factor α (PPAR α) play a pivotal role in early alcoholic liver injury [82]. One of the most well studied transcription factors is NF κ B which has a pivotal role in controlling the expression of pro-inflammatory cytokines in monocytes and macrophages. However the expression of NF κ B displayed a surprising response to ethanol treatment depending on whether the treatment is acute or chronic. Differences have been noted in the ability of acute and chronic alcohol exposure to modulate monocyte/macrophage activation. Acute alcohol exposure is associated with LPS-induced anti-inflammatory effects in monocytic cells in humans both *in vivo* and *in vitro* [83-85]. By contrast chronic alcohol induces the expression of TNF α in human and mouse models [69, 86, 87]. This switch between anti-inflammatory and pro-inflammatory responses is linked with changes in IL-1R-associated kinase-monocyte (IRAK-M), a negative regulator of LPS

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2 signalling and leads to decreased downstream activation of LPS signalling cascade culminating in
3 impaired NFκB DNA binding and transactivation and finally TNFα production. In contrast chronic
4 alcohol exposure switches the anti-inflammatory to a pro-inflammatory response by decreasing
5 IRAK-M and increasing IRAK-1 and IKK kinase activation followed by enhanced NFκB DNA binding,
6 transactivation and ERK kinase activation and TNFα production [88]. Other transcription factors such
7 as Activator Protein-1 (AP1), PPARγ and Egr1 have also been shown to be modulated by exposure to
8 alcohol [89, 90, 91].
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13 **Animal models of DILI associated inflammation**

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15 There is a lot of data to support the role of inflammation in the progression of human liver
16 disease as has been described for viral hepatitis and alcohol liver disease. Currently sufficient data to
17 support an unequivocal role for inflammation in DILI is lacking and in many cases mechanistic details
18 are unknown. Evidence to support the hypothesis that DILI triggers the activation of the innate
19 immune system within the liver has been obtained from experimental *in vivo* models. The
20 advantages of investigating inflammation in animal models of DILI are the stable genetic, molecular
21 and immunological backgrounds of inbred strains. This lack of variation enables a more complete
22 exploration and determination of possible inflammatory mechanisms contributing to the overall
23 pathophysiology. However, although animal models are useful tools with which to investigate DILI
24 and the involvement of inflammation caution must be shown in the translation of these results to
25 human DILI.
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33 **Inflammation in an acetaminophen (APAP) model of DILI**

34 A widely studied model of DILI has been APAP-induced liver injury (AILI) in mice (Table 2).
35 Evidence suggests that the initial hepatocellular damage caused by NAPQI may lead to the release of
36 cellular contents known as damage associated molecular patterns (DAMPs). These include HMGB-1
37 or Heat shock proteins (HSP) which act as signals which can activate cells of the innate immune
38 system which reside in the liver. This in turn leads to hepatic infiltration of inflammatory cells which
39 may contribute to the progression of liver injury by producing pro-inflammatory mediators such as
40 cytokines and chemokines as well as reactive oxygen and nitrogen species (Figure 1, [92]).
41 Neutrophils accumulating in necrotic areas of the liver after APAP overdose were first recognised by
42 Mitchell et al. [93] but the relevance of this accumulation to the pathophysiology is still unclear [94].
43 A study in C3Heb/FeJ mice revealed that neutrophils accumulate in the liver shortly after hepatic
44 injury but there was no evidence of neutrophil activation. Additionally, antibodies targeted at these
45 cells did not protect against AILI [95]. In a rat model of AILI, a significant reduction in neutrophil
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2 accumulation showed no protection of liver injury [96]. These findings are in contrast to other
3 models in which neutrophils are involved in the course of injury where pre-treatment with an anti-
4 neutrophil antiserum attenuated AILI in rats [97].
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8 Other studies have demonstrated that mice treated with gadolinium chloride to inactivate KCs
9 [98, 99] and liposome/chlodronate to deplete KCs [99, 100] are protected against APAP toxicity.
10 Though reports suggest that KC depletion confers protection at early times after APAP treatment
11 [100]but can lead to more severe injury at later time points [99]. The use of an anti-NK1.1
12 monoclonal antibody to deplete NK and NKT cells significantly protected mice from APAP toxicity
13 probably due to the reduction in the levels of the pro-inflammatory cytokine IFN- γ [101]. However as
14 has been observed with KC depletion, there are conflicting reports suggesting that NK/NKT cells play
15 little role in the overall outcome of liver injury in the mouse model of APAP hepatotoxicity [102,
16 103]. This has been supported by a recent study that suggested NK and NKT cells only contributed to
17 liver injury when dimethyl sulfoxide (DMSO) was used to solubilise APAP [103]. Given the conflicting
18 data available on neutrophils and KCs it is clear that more studies are required to determine their
19 precise role in DILI.
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27 The involvement of various pro-inflammatory mediators such as TNF- α [104-106], IL-1ra [107],
28 IFN- γ [101, 108], in promoting tissue damage have been demonstrated whereas other cytokines
29 such as IL-10 and IL-4 [109] have been found to be hepatoprotective in models of APAP
30 hepatotoxicity. Receptors involved in promoting an inflammatory response such as IL-1 receptor
31 [106], p55 TNF- α receptor-1 [105], Toll-like receptors TLR4 and -9 [110, 111] and receptor for
32 advanced glycation end-products (RAGE) [112] have been found to be important in promoting an
33 inflammatory response and subsequent pathogenesis.
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Table 2: Summary of inflammatory models used in APAP-induced hepatotoxicity

| References | Inflammatory model(s) | Reported effect |
|------------|--|---|
| [99, 100] | KC depletion | Protection against liver injury at early time points, more susceptible at later time points |
| [113] | Neutrophil depletion | Significantly protected against liver injury |
| [101] | NK/NKT cell depletion | Significantly protected against liver injury |
| [106] | Anti-TNF- α /IL-1- α /IL-1ra | Increased susceptibility to liver injury |
| [105] | Anti-TNF- α , TNF-p55 receptor 1 ^{-/-} mice | Reduced mortality and liver injury |
| [120] | TNFR1 ^{-/-} mice | Increased susceptibility to liver injury |
| [108] | IFN- γ ^{-/-} mice | Significantly protected against liver injury |
| [117] | IL-6 ^{-/-} mice | Increased susceptibility to liver injury |
| [109] | IL-10/4/6 ^{-/-} mice IL-10/4 ^{-/-} mice | Significantly protected against liver injury Increased susceptibility to liver injury |
| [118] | IL-13 ^{-/-} mice | Increased susceptibility to liver injury |
| [111] | Anti-TLR9, TLR9 ^{-/-} , Nalp3 ^{-/-} mice | Reduced mortality and liver injury |
| [112] | Soluble RAGE | Reduced mortality and liver injury |
| [115] | LPS | Increased susceptibility to liver injury |

Comparison of the inflammatory response in animal models of acetaminophen-induced liver injury

Several different models of DILI in which inflammation has been modulated are being investigated in an attempt to dissect the processes of inflammation from those on DILI. NK and NKT cells were found to play a critical role in the severity and progression of AILI (500mg/kg, i.p) by secretion of cytokines and recruitment of inflammatory cells (mainly neutrophils) into the liver [101, 113]. An increase of hepatic neutrophil accumulation as early as 4-6 hours with a significant 3-fold increase by 24 hours was observed in APAP-treated mice with an associated increase in mRNA levels for cytokines TNF- α and IFN- γ [101]. Depletion of NK and NKT cells (using an anti-NK/NKT cell antibody or an anti-neutrophil antibody) inhibited liver injury with markedly reduced accumulation of neutrophils in the liver. Subsequent depletion of neutrophils also significantly protected mice

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2 against AILI. However, in a recently published fed CD-1 mouse model of AILI, no evidence of hepatic
3 inflammatory cell infiltration was observed over a 24 hour time course [114].
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6 Non-toxic doses of APAP (100-400mg/kg, i.p) administered 2 hours after a dose of LPS (44×10^6
7 EU/kg) to fasted C57BL6 mice have been shown to produce a leftward shift of the dose-response
8 curve for AILI [115]. Application of LPS led to significantly greater TNF- α production than APAP alone.
9 With APAP treatment alone there was a modest, sustained accumulation of neutrophils from 3 - 24
10 hours after 300mg/kg APAP. However neutrophils were not apparent in livers of mice treated with a
11 non-toxic dose of APAP (175mg/kg) but significant accumulation occurred when this dose was co-
12 administered with a non-injurious dose of LPS. Two hours after treatment with LPS (0 hours after
13 APAP), serum TNF- α levels were large and rapidly decreased thereafter, confirming that the
14 inflammatory response observed in this model was due to LPS [115]. These observations support the
15 hypothesis that inflammation induced by LPS and associated elevated levels of TNF- α increase
16 sensitivity to AILI.
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24 Fasted transgenic C57BL6 mice lacking anti-inflammatory mediators such as IL-10, IL-4, IL-6, MIF
25 and IL13 dosed with APAP (200-300mg/kg, i.p) displayed increased susceptibility to AILI [109,116-
26 118]. Neutrophils were found to accumulate to a greater extent in IL13 KO mice than wild-type
27 treated with APAP with the level of TNF- α , IL-6 and IFN- γ increased at 4 hours in WT and IL-13 KO
28 APAP-treated animals compared to saline-treated controls [118]. These data suggest a
29 hepatoprotective role for anti-inflammatory mediators in these models and that the balance
30 between the Th1 and Th2 response is an important determinant for AILI. Susceptibility to AILI was
31 compared between two mouse strains, C57BL6 (more susceptible to AILI and which develop
32 predominantly a Th1 response) and BALB/c (less susceptible to AILI and which develop a Th2
33 response). 24hr after exposure to APAP C57BL6 mice produce high levels of TNF- α (pro-
34 inflammatory) whereas levels in BALB/c mice show no difference. On the other hand IL-6 (anti-
35 inflammatory) levels were higher in BALB/c than C57BL6 24 hours after administration. These data
36 support the hypothesis that AILI is associated with a Th1-dominant response in Th1/Th2 cytokine
37 balance and TNF- α may play a role in toxicity [117].
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46 However, the role of TNF- α remains controversial with contradictory reports suggesting that
47 TNF α is involved in pathogenesis of DILI [105-106]; is not involved in toxicity [104-119] or it has a
48 role in defence against toxicity and repair [120-121]. Data suggests that like most cytokines the
49 effects of TNF α in liver toxicity are pleiotropic with other cytokine networks being involved and it is
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the interplay between these different cytokine cascades that determine the nature of the inflammatory response associated with toxicity. IL1 α [106] and INF γ [108] have been shown to play a pathogenic role in APAP-induced hepatotoxicity whilst IL-6 [117] and IL10 [116] play a protective role. Furthermore, the understanding of the regulatory mechanisms in the context of DILI that control the potential for DAMPS or cytokines to activate immune cells and initiate an inflammatory response require further investigation. For example, recent evidence suggests that the caspase-dependent oxidation of HMGB1 is critical for inhibiting its immune stimulatory potential following apoptosis [16].

As well as APAP, other model hepatotoxins have been used to investigate the role inflammation plays during drug-induced liver injury (Table 3). Given the complexity of interplay between chemically mediated toxicity and inflammation, and the differences observed between strains of mice, species and *in vivo* model pre-treatment further investigation is required to define the precise role a cytokine has in response to the injury or as part of the inflammatory response. Once this has been defined then the value of the cytokine as a biomarker can be assessed.

Table 3: Inflammation in other models of DILI

| DILI Model | Inflammatory factors investigated | References |
|---------------------|-----------------------------------|------------|
| LPS | Neutrophils, TNF- α , KCs | [122-124] |
| EtOH | Kupffer cells, TNF- α | [125-128] |
| Halothane | Macrophage inhibitory factor | [116] |
| Cocaine + LPS | Kupffer cells, nitric oxide | [129, 130] |
| Galactosamine + LPS | TNF- α , TNF-p55 receptor | [131, 132] |

Cytokine expression in clinical studies of acetaminophen-toxicity

As has been discussed many studies have investigated the role of cytokines in animal models. Some of these cytokines have been reported to have hepatoprotective effects in animal models and several studies have been performed to investigate cytokine expression patterns in serum samples from different patient cohorts. In a study of 111 patients (90 female; mean age 13.6 years) plasma concentrations of IL6, IL8, IL10 and Monocyte chemoattractant protein 1 (MCP1) were analysed by enzyme-linked immunosorbent assay. Patients were stratified by toxicity severity as defined by the maximal values of hepatic transaminase elevation. Levels of IL6, IL8 and MCP1 were elevated in patients with elevated serum alanine aminotransferase and MCP1 expression had the strongest association with toxicity [133]. Other studies have found associations between elevated

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expression levels of IL8 [134, 135]; IL6 [136] and acetaminophen-induced hepatotoxicity. While the results from these studies indicate that inflammation and cytokines are associated with the progression of late stage toxicity interpretation of the data is often limited by the absence of pre-dose data, a lack of samples from early in the process of drug-induced injury and the small number of samples usually obtained in the clinic.

Several other studies have investigated the expression of various cytokines associated with various forms of acute liver failure (ALF). In a mixed cohort of patients with ALF and acutely decompensated cirrhosis it was found that not only was the degree of systemic inflammation, as measured by IL6 and TNF α , important for determining outcome but that the magnitude of the anti-inflammatory response was also important. In the whole cohort, IL10 (regarded as an anti-inflammatory cytokine) levels were found to predict outcome as accurately as IL6 [137]. However these patients are experiencing acute liver failure and while expression of the cytokine is linked with disease progression the patient cohorts are characterised by end stage severe liver failure and it is therefore difficult to relate cytokine expression patterns with the earliest stages of injury when intervention is required to prevent toxicity from developing further.

Recently a couple of studies have been reported which attempted to address the issues surrounding patient sampling by treating volunteers with a bolus of acetaminophen under controlled conditions. This has allowed the determination of a pre-acetaminophen treatment baseline for parameters and accurate timing of sampling post-acetaminophen dose. DNA microarrays and serum metabolomic methods were employed to characterise changes in the transcriptome and metabolome in serial peripheral blood (PB) samples obtained from six healthy adults treated with a 4-g bolus of acetaminophen and from three receiving placebo. Treatment did not cause liver toxicity as assessed by traditional liver chemistries. It was found that a single 4-g bolus of acetaminophen produced a transcriptomic signature in PB cells characterised by down regulation of oxidative phosphorylation genes accompanied by increased serum lactate. Similar gene expression changes were observed in rats and several patients after consuming hepatotoxic doses of acetaminophen [138]. While this study is exciting as it begins to allow an insight into the progression of acetaminophen induced liver injury in a controlled setting, it also highlights the challenges faced in identifying novel biomarkers for DILI that can be applied in the clinic. The changes reported in this paper occurred 48 hours after the acetaminophen was administered to volunteers and are therefore likely to be downstream of liver injury. The authors also recognise that the mechanism by which liver toxicity affects peripheral blood cells is unclear, are the changes observed direct or indirect effects?

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4 In a separate study it was shown that urine metabolite profiles from healthy adults each
5 receiving 4g of acetaminophen per day for 7 days profiles obtained before start of treatment were
6 not sufficient to distinguish which of the subjects would develop mild liver injury. However profiles
7 obtained shortly after the start of treatment but prior to ALT elevation, could distinguish responders
8 from non-responders [139]. This data suggests that an early-intervention pharmaco-metabolomics
9 approach could be used to identify potential toxicities early, however much more work is required to
10 validate this approach. However in both these studies it is interesting to note that few, if any, of the
11 changes described were associated with cytokines. Analysis of liver chemistries showed that liver
12 toxicity had not been caused by the dose of paracetamol used, therefore it indicates supports the
13 hypothesis that inflammation and cytokines are involved only after overt liver damage has occurred.
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19 Immunological involvement in idiosyncratic DILI – the example of halothane

20 Lessons from animal studies have identified the potential role of the innate immune
21 response in dictating the overall response during APAP hepatotoxicity; however, this stills needs to
22 be proven in man. While the role of immune cells, and by extension cytokines, remains unclear
23 other approaches are been taken to identify biomarkers. Some of the characteristics of idiosyncratic
24 DILI reactions appear to have an immune component and as many autoimmune functions appear to
25 be mediated by TH17 cells, it was hypothesised that IL17 may play a role in iDILI [140]. Expression
26 patterns of cytokines were examined in the serum of 39 patients with acute liver failure (ALF) due to
27 IDILI and compared the values with those from 21 patients with acetaminophen-induced ALF and 10
28 patients with viral hepatitis-induced ALF. IL-17 levels were found to be elevated in a similar number
29 of patients IDILI and acetaminophen-induced ALF and occasionally in patients with viral hepatitis.
30 There was overlap in the level of expression of IL-21 in patients with iDILI and AILI. Auto-antibodies
31 were more frequent in patients in the IDILI group but were absent in most patients. These data
32 provide a picture of the cytokine/chemokine profile in patients with various types of ALF. The
33 pattern varies from patient to patient and not specifically by etiology. This suggests that different
34 underlying disease mechanisms may be at play in different individuals, even among those
35 demonstrating injury from the same drug. Since cytokines may originate from more than one type of
36 cell, interpretation of results of cytokine assays remains difficult in complex disease settings.
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47 However it is certainly possible that an adaptive immune response may play a role in cases
48 of human DILI. Certain drug reactions have clinical hallmarks of an immunological mechanism, the
49 liver alone may be involved or liver injury may be part of a more complex hypersensitivity syndrome.
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2 Unlike with APAP, where the initiation of hepatotoxicity is dependent upon the intrinsic chemistry of
3 the molecule, these so called idiosyncratic hepatotoxicity reactions represent a function of an
4 individual patient's biology (Table 4).
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6 The inhalation anaesthetic halothane is the best-studied drug causing an immunoallergic
7 response in a subset of patients. The response ranges from asymptomatic rises in transaminases to
8 fulminant hepatitis, which is rare but serious. Most of the patients recorded in the literature with
9 immunoallergic hepatitis had more than one exposure [141]. Antibodies have been detected in such
10 patients that recognise autoantigens and neoantigens created by trifluoroacetylation (TFA) of
11 hepatic proteins. Pre-incubation of halothane pre-treated rabbit hepatocytes with sera of patients
12 with halothane-induced fulminant hepatitis increased susceptibility to cytotoxicity of lymphocytes *in*
13 *vitro* [142]. It is likely drug specific T cells play a role in the pathogenesis of hepatocyte injury but
14 direct evidence is lacking. Halothane is metabolised by CYP450 2E1, like APAP, but forms a
15 chemically reactive acyl halide and bioactivation to the acyl halide is substantial as it is the only
16 metabolic route available. It is hypothesised that the formation of the acyl halide triggers an immune
17 response and that mild and severe immune responses are likely to have this common initiation
18 mechanism. Evidence for this concept of an immune trigger is both direct and indirect, with drug
19 specific antibodies indicative that the drug has initiated an immune response in affected patients
20 and newer metabolically inert inhaled anaesthetics being rarely associated with hepatotoxicity.
21 Target proteins modified by the acyl halide have been identified with the principal chemical
22 modification trifluoroacetylation of lysine residue [143-146]. Chemical modification of protein(s) is
23 associated with the immune response observed with halothane hepatitis, however, there is no
24 conclusive evidence that the antibodies mediate liver injury. Antidrug antibodies have also been
25 observed with idiosyncratic drug reactions to Tienilic acid [147] and dihydralazine [148] but there is
26 no unequivocal evidence that DILI such as that caused by halothane is immune mediated. Most of
27 the information available is compatible with the hapten hypothesis where the drug undergoes
28 bioactivation in hepatocytes leading to drug-protein conjugate formation in the liver. The current
29 theory suggests that the occurrence of idiosyncratic drug hypersensitivity in man represents
30 interplay between a number of factors, which include the chemistry of the drug, drug metabolism
31 and the immune system of the patient (Table 4). Whether cytokines may be useful biomarkers
32 in such idiosyncratic cases is currently unknown as the data supporting immune involvement is
33 equivocal and the mechanistic details are unknown. However, if the hapten hypothesis is correct
34 what is clear is that the inflammatory response induced by chemical insult such as APAP overdose
35 compared to that of idiosyncratic DILI may be very different.
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Table 4: Idiosyncratic drug reactions with evidence of immune involvement (amended from [149])

| Drug Class | Drug Name | Is there evidence to support immune involvement? |
|-------------------------|---------------------------|--|
| Anaesthetic | Halothane | Yes |
| Analgesic | Diclofenac | Yes/No |
| | Sulindac | Yes |
| Antibiotic | Amoxicillin | Yes |
| | Erythromycins | Yes |
| | Minocycline | Yes |
| | Nitrofluantoin | Yes |
| | Rifampicin | Yes/No |
| Anti-convulsant | Phenytoin | Yes |
| | Phenothiazines | Yes |
| Anti-epileptic | Carbamazepine | Yes/No |
| Antihypertensive | Dihydralazine | Yes |
| | Methyldopa | Yes |
| | Tienilic acid | Yes |
| Anti-thyroid | Propylthiouracil | Yes |
| Depression | Tricyclic antidepressants | Yes |
| Gout | Allopurinol | Yes |
| Hypertension | ACE inhibitors | Yes |

Conclusions and perspectives

An analysis of the role of inflammation in human liver disease and in the *in vivo* models described offer insights into the potential role of inflammation in human DILI. Given the role of cytokines in modulating the inflammatory response changes in patterns of cytokine expression certainly show promise as potential biomarkers of DILI. However, the *context* of cytokine expression will determine their utility as biomarkers. This context will be determined by understanding the processes within DILI and the associated inflammatory cascade in which the cytokine has a role (Figure 1). One key question is whether the inflammatory response precedes the injury or is a consequence of the injury (Figure 2). Also, inflammation is associated with a lot of confounding factors experienced in the clinic (as previously described) therefore much more data is required in order to support the context of a given cytokines' qualification for use as a biomarker and the proposed application of that biomarker. Clearly prior to selecting a given cytokine as a biomarker it

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2 is important to accurately phenotype DILI and to have a thorough understanding of the processes
3 and mechanisms by which injury occurs.
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6 As discussed the balance of pro and anti-inflammatory cytokines is thought to play a
7 significant role in dictating the overall balance of hepatic injury exacerbation or regeneration, in
8 particular for DILI characterised as idiosyncratic in nature (Figure 2). The majority of animal models
9 of DILI are exposed to profound, often lethal levels of chemical stress with changes in the expression
10 of cytokines such as TNF α , HMGB-1, IL6, IL10 and IL1 β [16, 18-20]. Therefore animal model data may
11 be misrepresentative of the majority of cases of liver injury observed clinically. The promotion of
12 inflammation may be the most significant consequence of chemical stress in the exacerbation of
13 DILI, in which case, cytokines remain an attractive option as biomarkers of DILI to inform the
14 mechanism of pathology, sensitively screen for hepatic injury and predict at risk patients.
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21 Even when a cytokine has been shown to be a candidate biomarker the benefit from use of a
22 novel biomarker in terms of safety efficacy and or cost must be clearly established. Biomarkers must
23 be sensitive and specific and this requires accurate definition of the context for which a biomarker
24 should be qualified [150]. Context and qualification for new biomarkers are assessed relative to
25 current biomarkers. If the sensitivity and specificity of current biomarkers are not perfect relative to
26 a specific end point, the context and qualification of new biomarkers may not be accurately
27 established. This is a particularly difficult problem if current biomarkers have pseudo-quantitative,
28 qualitative, or subjective metrics associated with them. Even the data available for the gold standard
29 of DILI (ALT) is continually reviewed to ensure that the context of its use is as accurate as possible
30 [151].
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37 Much remains to be understood about the role of inflammation in DILI and cytokines in
38 controlling this inflammatory response. Cytokines are interesting candidates for novel biomarkers as
39 they are relatively accessible (by blood sampling) and quantifiable. However without fully
40 understanding the role of inflammation in DILI they may be limited in their use to being diagnostic of
41 the type of injury that has occurred and maybe prognostic of outcome (recovery from DILI, cirrhosis,
42 acute liver failure). Intracellular components released by damaged hepatocytes, although
43 inaccessible and currently difficult to quantify may be better biomarkers for the prognosis of
44 whether an injury will occur (Figure 1). However, further investigation is required to understand the
45 impact of chemical stress on cellular physiology and the release of intracellular components in
46 response to injury as well as the development of assays sensitive enough to accurately detect them.
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Figure Legends

Figure 1 Paracetamol-induced hepatotoxicity: a multistep/multicellular process

The multi-step process and multi-cell type involvement during acetaminophen-induced hepatotoxicity. Acetaminophen (APAP) hepatotoxicity is dependent upon the metabolic activation by CYP450 to the reactive metabolite NAPQI. Following saturation of the phase II conjugation detoxification pathways, NAPQI becomes covalently bond to hepatic protein (in particular mitochondrial protein) which leads to cell death. Cell death can proceed through either apoptosis or necrosis depending upon the bio-energetic status of the cell. So called DAMPs (e.g HMGB-1) are released from necrotic cells which hold the potential to activate the innate immune response. Evidence from animal models suggests that the overall extent of hepatic damage is dictated by the balance between the production of pro and anti-inflammatory cytokines. The dependency upon the multiple steps has been elucidated experimentally in vitro and in vivo from CYP450 dependence (inhibitor; ABT), glutathione level (NAC; N-acetyl-cysteine), mitochondrial permeability transition (CsA; cyclosporine), and cellular ATP status (dietary restriction) to caspase activation (inhibitor; Z-VAD.fmk)

Figure 2 Inflammatory etiology of acute liver failure

Acute liver failure stimulated by a variety of inducing agents leading to the development of the pathology often share common pathways through the activation of pro-inflammatory pathways. Initial necrotic cell death from reactive metabolites, drug accumulation, diet, alcohol or viruses can lead to the release of DAMPs which in turn activate the primary and secondary inflammatory cascades. Underlying hepatitis can influence an individual's susceptibility to hepatotoxicity. Understanding the mechanisms involved from the study of liver diseases or the particular serum cytokine finger print when applied to DILI may help to predict susceptible individuals or patients likely to develop severe hepatotoxicity

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