Novel Liver and Kidney Biomarkers

For Improved Drug Safety Research

- Biomarkers K18 & ccK18 are recommended to study drug hepatotoxicity[1, 2, 4]
- α-GST and ccK18 are excellent markers of liver fibrosis and steatosis in NAFLD & NASH research
- Urinary α-GST & π-GST are validated markers for drug-induced kidney toxicity[1, 3]
- Urinary collagen IV is a kidney biomarker for glomerular damage

Novel Liver and Kidney Biomarkers

Liver and kidney injury

Researching liver and kidney injury in clinical studies and drug development

A. Clinical Studies

With the increasing prevalence of obesity and metabolic syndrome an increase of non-alcoholic fatty liver disease (NAFLD) is obvious. The challenge is to find better methods of studying NAFLD and its progressive and chronic form NASH (non-alcoholic steatohepatitis). Similarly, renal diseases are an expanding problem and again the challenge is to find better methods to study kidney disease, like acute kidney injury (AKI).

B. Drug Development

Liver and kidney toxicity are important causes of late stage drug failure and post-launch warning texts. Reasons for this include the shortcomings of current toxicology biomarkers that lack sufficient sensitivity and specificity. The challenge is to find biomarkers with known origins and mechanisms of release that can improve the detection and of potential toxicity, potentially saving time and costs in drug development.

Toxicology testing is an important and expensive part of drug development. Predicting which drugs will prove toxic to the liver or kidney is an important aspect during drug development in order to reduce the risk of failure of new medicines. Hepatotoxicity and nephrotoxicity are important causes of post-launch withdrawal of pharmaceuticals and an important cause of Adverse Drug Reactions (ADRs). Drug-induced liver injury (DILI) now ranks as a leading cause of liver failure and transplantation in western countries. Similarly, drug-induced kidney injury (DIKI) contributes up to 60% of all cases of acute renal failure in hospitalized patients, a serious problem considering the high morbidity and mortality associated with Acute Kidney Injury (AKI).

An important reason for compounds failing late during pre-clinical phase is that current tests used to detect renal and hepatic injury have shortcomings. For liver injury, traditional biomarkers alanine aminotransferase (ALT) and aspartate aminotransferase (AST), have several limitations. In the course of testing a therapeutic for potential to cause liver injury, AST/ALT increases are commonly observed in the absence of evidence of injury to tissue, and, conversely, sometimes do not increase even when tissue injury is observed. In clinical settings, AST/ALT levels frequently do not increase even when liver injury is evident. For example, up to 25 – 30 % of subjects with fibrosis liver damage may have normal transaminase levels. Liver biopsy is still the gold standard for the detection of liver damage. This invasive examination method is limited by sample errors (e.g. inhomogeneous liver damage) and the risk of clinical complications.

Similarly, the common renal biomarker test, serum creatinine, is known to be a late, insensitive, indicator of renal glomerular function while most nephrotoxins affect the renal tubules. Therefore, European and USA initiative programs (the Innovative Medicines Initiative (IMI) SAFE-T consortium [1] and C-Path's Predictive Safety Testing Consortium [2]) validated new safety biomarkers to:

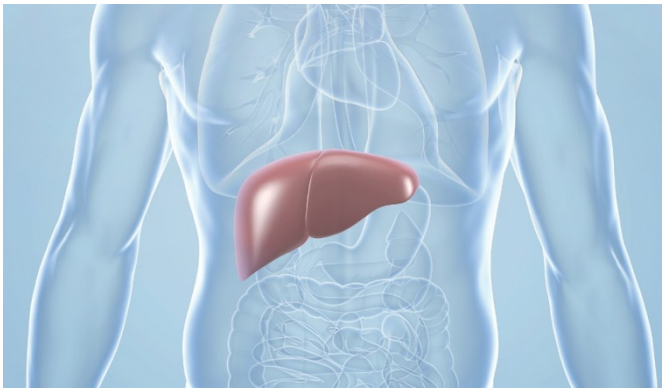
- Make better regulatory decisions in drug R&D.
- Detect, assess and monitor drug-induced kidney and liver injury in humans
- Diagnose disease in clinical practice

Using biomarkers with known sources and mechanisms of release can increase the specificity and sensitivity of organ damage testing. This can eliminate compounds with an increased risk of adverse reactions early from the drug discovery process and improve the monitoring of subjects with suspected liver or renal disease.

1 The European Innovative Medicines Initiative (IMI) SAFE-T consortium and USA C-Path's Predictive Safety Testing Consortium, a program run in partnership with the Food and Drug Administration (FDA.
2 Letter of support for drug-induced liver injury (DILI) biomarker, 30 September 2016, EMA/423870/2016
3 Letter of support for drug-induced renal tubular injury Biomarker(s), 14 December 2016, EMA/715025/2016

Biomarkers for liver injury

Liver fibrosis, cirrhosis, inflammation and steatosis are major features of acute and chronic liver diseases, such as viral hepatitis (HBV, HCV), autoimmune or metabolic liver diseases, alcoholic fatty liver disease (AFLD), non-alcoholic fatty liver disease (NAFLD) and steatohepatitis (NASH).



Detecting potential hepatotoxicity is important during drug development in order to reduce the risk of late stage failure of compounds and to reduce the risk of drugs showing unwanted hepatic effects. To mitigate the problems with standard liver tests, The European Innovative Medicines Initiative (IMI) SAFE-T project has validated liver injury biomarkers for application in the regulatory decision-making process in clinical drug development and as potential clinical tests. C-Path's Predictive Safety Testing Consortium, a program run in partnership with the Food and Drug Administration (FDA), is validating biomarkers for similar reasons.

Conclusions are:

- SAFE-T concludes that total keratin 18, a marker of hepatocyte necrosis and caspase-cleaved keratin 18, a marker of hepatocyte apoptosis have potential as DILI biomarkers to provide additional information beyond the diagnostic value of ALT[3].

- FDA issued a letter of support for Keratin 18 as a promising biomarker to address DILI[4].

Note: Serum Alpha Glutathione S-Transferase (α-GST), a rapidly released leakage marker upon hepatocyte damage, is another attractive option for addressing DILI.

Liver injury biomarker	Indicative for	Cell culture measurements
Caspase - cleaved Keratin 18 (ccK18: M30 Elisa)	Hepatocyte apoptosis	√ (2D and 3D)
Keratin 18 (cleaved and uncleaved: M65 Elisa)	Hepatocyte apoptosis and necrosis	√ (2D and 3D)
Alpha Glutathione S-Transferase (α GST)	Hepatocyte damage	√ (2D and 3D)[5]
Pi Glutathione S-Transferase (π GST)	Bile duct damage	√
Collagen IV (serum)	Increased collagen deposition	
Hyaluronic acid (HA)	Liver fibrosis and loss of liver function	√ (2D)

Table 1. *Type of information provided by various liver injury biomarkers*

Novel liver injury biomarkers provide useful information about the nature of the liver damage and have practical utility in following situations:

Drug development and preclinical studies

- Drug development toxicity studies using liver cell culture models[5].
- Toxicity studies in humans.

Other research areas

- Liver fibrosis and cirrhosis
- NAFLD and NASH progression studies
- Diagnosis of Hepatocellular carcinoma studies
- Immunosuppressive therapy in autoimmune hepatitis studies
- Graft versus host disease studies
- Environmental toxicants induced liver injuries

3 SAFE-T consortium - Summary Data Package Novel clinical biomarkers of Drug-Induced Liver Injury, September 2016.
4 Letter of Support for Drug-Induced Liver Injury (DJU) Biomarker(s), FDA July 25, 2016.
5 α-GST Release as a Predictive Marker of Drug Induced Hepatotoxicity Utilising 3D InSight™ Liver Microtissues. Application note (2013).

Available assay kits for liver injury

Caspase-Cleaved Keratin 18 (CCK18)[6]

- Keratin 18 (K18) is an intermediate filament required for maintenance of the cytoskeletal architecture.
- Expressed by epithelial cells like hepatocytes and cleaved by apoptosis enzymes, so called caspases.
- ccK18 serves as a highly specific marker for hepatocyte apoptosis.

Keratin 18 (Cleaved and Uncleaved)[7]

- Keratin 18 (K18) is an intermediate filament required for maintenance of the cytoskeletal architecture.
- Expressed by epithelial cells like hepatocytes and cleaved by apoptosis enzymes, so called caspases.
- K18 serves as a highly specific marker for hepatocyte cell death (necrosis and apoptosis).

α-gst (Alpha Glutathione S-Transferase)

- Constitutively expressed cytosolic protein involved in cellular detoxification.
- Localized to hepatocytes.
- Rapidly released (Leakage) when hepatocytes are damaged.

π-gst (Pi Glutathione S-Transferase)

- Constitutively expressed cytosolic protein involved in cellular detoxification.
- Localized to bile duct cells, rapidly released (Leakage) when bile duct cells are damaged.
- π-GST can be measured in plasma as it is expressed and leaked from damaged bile duct epithelia. π-GST cannot be measured in serum as platelets are a potential source of non-hepatic π- GST.

Collagen IV

- Structural protein of extra cellular matrix, abundant component of the basement membrane.
- The first collagen to be deposited when membranes are deposited.
- Biomarker of active collagen deposition in liver.
- Excellent biomarker of liver scarring and fibrosis.

Hyaluronic Acid (HA)[8]

- Major component of connective tissues; about one fourth is found in the skeleton and its supporting structures like ligaments and joints.
- Excellent biomarker to research status and development of fibrosis

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6 See TECOmedical Bulletin - M30/NASH (Cytokeratin 18)
7 See Bulletin – M65/liver toxicity (Cytokeratin 18)
8 See TECOmedical Bulletin – Hyaluronic Acid Biomarker for Diagnosis and Monitoring of Liver Fibrosis & Cirrhosis

Biomarkers for kidney injury

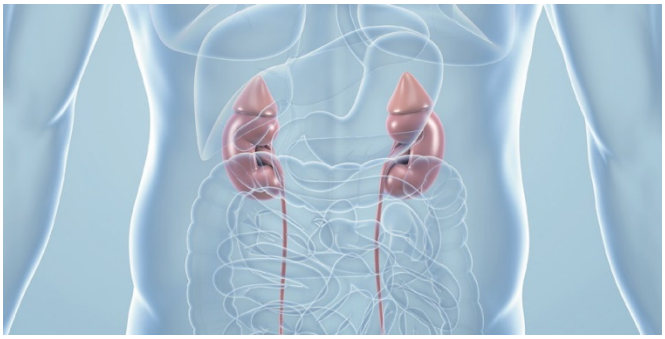
Studying drug-induced Kidney injuries is an important aspect during drug development in order to reduce the risk of failure of new medicines. The European Innovative Medicines Initiative (IMI) SAFE-T project has validated novel kidney injury biomarkers C-Path's Predictive Safety Testing Consortium, a program run in partnership with the Food and Drug Administration (FDA) has validated novel biomarkers for the same reasons as IMI-SAFE-T. Conclusions are:

A. Both, European and USA projects indicate that urinary α-GST and π-GST are sensitive and specific biomarkers of renal tubular injury and validated biomarkers for kidney toxicity.
B. EMA issued a Letter of Support to SAFE-T and C-Path to encourage further development of Urinary α-GST (and other markers) to assess drug-induced kidney injury (DIKI)[9].

Over 50% of kidney function is lost before Acute Kidney Injury (AKI) is detected by elevated serum creatinine levels (Figure 1). Novel kidney injury urinary biomarkers can detect AKI as early as 4-6 hours following the initial kidney injury (serum creatinine: 24-48 hours; Figure 1,2 and Table 2). Use of these kidney biomarkers allows early and site-specific prediction of kidney injury in:

Drug Development and Pre-Clinical Studies

- Toxicity studies
- Drug development using kidney cell culture models



Additional Research Areas

- Acute kidney failure
- Contrast fluid and drug induced damage
- Damage induced by environmental toxins
- Kidney transplant graft rejection
- Chronic kidney disease and diabetes

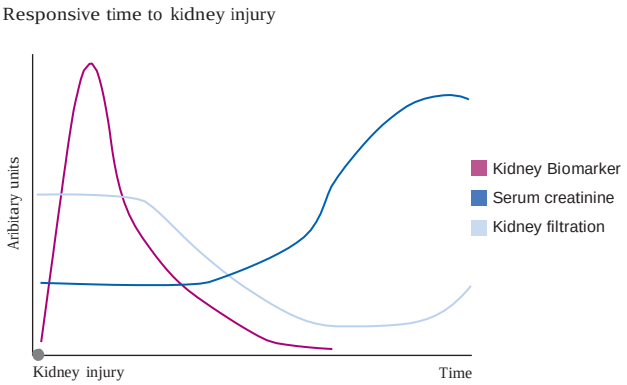


Fig. 1
Response of novel renal injury biomarkers and serum creatine following renal injury artistic impression.

Table 2.
Type of information provided by various kidney injury biomarkers

Kidney Biomarker	Tissue specificity	Indicative for	Response time following injury*	Cell culture measurements
Alpha Glutathione S-Transferase (α GST)	Proximal tubule	Necrosis	> 4- 6 hours	√ (2D and 3D
Pi Glutathione S-Transferase (π GST)	Distal tubule	Necrosis	> 6 hours	√
Collagen IV	Glomerulus	glomerular damage	Chronic deposition	progression of renal fibrosis.
Endostatin	Glomerular and tubular basement membranes	progression of renal fibrosis	Chronic deposition	progression of renal fibrosis.

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9 EMA Letter of support for drug-induced renal tubular injury Biomarker(s), 14 December 2016. EMA/715025/2016

Available assay kits for kidney injury

For proximal tubular damage

α-gst –urine (Alpha Glutathione s-Transferase)

- Constitutively expressed cytosolic protein involved in cellular detoxification.
- Localized to epithelial cells of the proximal tubules.
- Rapidly released (Leakage) when cells are damaged.

For distal tubular damage

π-gst - urine (Pi Glutathione S-Transferase)

- Constitutively expressed cytosolic protein involved in cellular detoxification.
- Localised to epithelial cells of the distal tubules.
- Rapidly released (Leakage) when cells are damaged.

For progression of renal fibrosis

Collagen IV – urine

- Structural protein of extra cellular matrix, abundant component of the glomerular basement membrane and mesangial matrix.
- Increased deposition in chronic renal disorders.
- Urinary levels correlate to the degree of mesangial expansion.
- Useful for researching renal disease progression in type 2 diabetic patients.

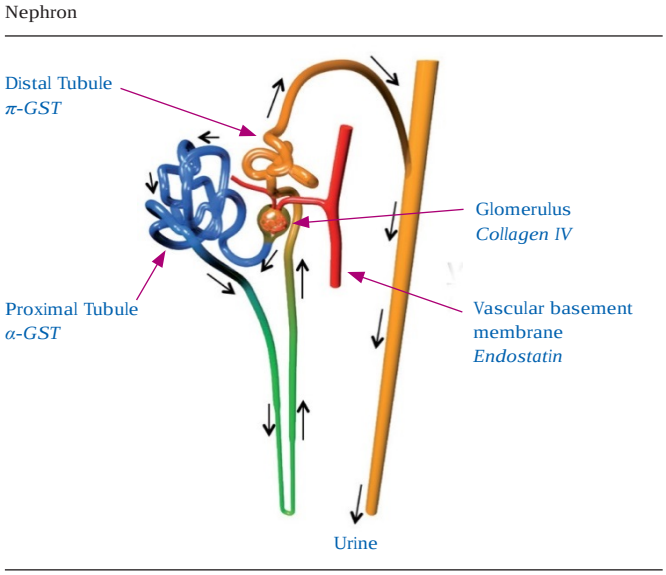

Fig 2.
Nephron specificity of kidney injury biomarkers

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Product information liver biomarker

M30 Apoptosense® ELISA (PEVIVA®) – Cat. No.: P10011	
Sample type	Human serum or plasma (EDTA, citrate, heparin plasma, cell lysate or cell culture supernatant from CK18 positive (epithelial) apoptotic cells or tissues.
Sample preparation	Whole blood stable for 48 hours at room temperature (Data available). Serum/plasma samples can be stored for long term at minimum -20°C. Repeated freeze-thawing procedures do not affect the reactivity of the samples.
Reference values	Serum of 200 blood donors median 94 U/L and 95th percentile 251 U/L. Normal <150 U/L Grey zone 150 – 200 U/L Elevated >200 U/L

M30 CytoDeath™ ELISA (PEVIVA®) – Cat. No.: P10900	
Sample type	Cell lysates or culture supernatants.
Sample preparation	Samples are stable up to one day at 2 – 8 °C, for at least 9 months at -20 °C, and for at least two years when stored at -80 °C . Avoid repeated freeze-thawing.
M65 EpiDeath® ELISA (PEVIVA®) – Cat. No.: P10040	
Sample type	Human serum or plasma (EDTA, citrate, heparin plasma, cell lysate or cell culture supernatant from CK18 positive (epithelial) apoptotic cells or tissues.
Sample preparation	If the assay is to be performed the same day, the samples can be stored at 2 – 8 °C. Samples are stable for at least 9 months at -20 °C, and for at least two years when stored at -80 °C.
Reference values	≤ 128 U/l Normal; 128 – 183 U/l Grey zone; > 183 U/l Elevated (if carcinoma is excluded).

M65® ELISA (PEVIVA®) – Cat. No.: P10020	
Sample type	Human serum or plasma (EDTA, citrate, heparin plasma, cell lysate or cell culture supernatant from CK18 positive (epithelial) apoptotic cells or tissues.
Sample preparation	Fresh samples are stable for up to two days at 2 – 8 °C, for at least 9 months at -20 °C; and for at least two years when stored at -80 °C.
Reference values	Median level 264 U/L with a range between 136 – 480 U/L. 95 th percentile 413 U/L. Based on the distribution the cut-off value for elevated K18 has been set at > 450 U/L.

α-GST, Human serum & plasma – Cat. No.: TE1056	
Sample type	Serum & Plasma.
Sample preparation	Centrifuge within 3 hours after collection. Samples can be stored at 20 – 25 °C for up to 48 hours, at 2 – 8 °C for up to one week or at -20 °C for >1 year. Repeated freeze thawing of samples should be avoided.
Reference values	0 - 12 µg/l.

Collagen IV, Human serum – Cat. No.: TE1053	
Sample type	Serum.
Sample preparation	Samples can be stored at 2 – 8 °C for one week, at -20 °C for 12 months. Repeated freeze-thawing of samples should be avoided.
Reference values	99 ± 23 µg/l (Mean ±1 SD, N= 180).

Hyaluronic Acid PLUS – TE1018-2 (CE)	
Sample type	Serum, EDTA-plasma and cell culture supernatant.
Sample preparation	Fasting blood collection.
Reference values	Values are dependent on age and gender and influenced by food intake and physical activity. The mean hyaluronic acid concentration is 36.7 ± 23.5 ng/ml. Based on these values a cut-off of 90 ng/ml has been defined.

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TECOmedical Group

Product information kidney biomarker

α-GST, Human urine Cat. No.: TE1056	
Sample type	Urine (Suggested initial dilution 1:2) with urine stabilizing buffer (TE1050).
Sample preparation	As soon as possible after collection sample should be diluted with urine stabilizing buffer (USB). Sample:USB 4:1. After the addition of USB, samples can be stored at 20-25 °C for up to 48 hours, at 2-8 °C for up to one week or at - 20 °C for >1 year. Repeated freeze thawing of samples should be avoided.
Reference values	0 - 29.0 µg/l (n = 120)

π-GST, Human urine Cat. No.: TE1085	
Sample type	Urine (Suggested initial dilution 1:2) with urine stabilizing buffer (TE1050).
Sample preparation	If the sample seems to contain blood, it must be centrifuged prior to processing and testing (see IFU for full instructions). Once clear of haemolysis contamination, sample should be diluted with urine stabilizing buffer (USB). Sample: USB 4:1. After the addition of USB, samples can be stored at 20-25 °C for up to 48 hours, at 2-8 °C for up to one week, or at -20 °C for >1 year. Repeated freeze-thaw cycles should be avoided.
Reference values	0 – 30 µg/l (n=132).

Collagen IV, Human urine Cat. No.: TE1054	
Sample type	Urine.
Sample preparation	If left standing Collagen IV can precipitate out of urine resulting in falsely low reported results. For this reason we recommend the use of Collagen IV sample collection tubes which ensure accurate reproducible measurement. After collection in this manner samples may be stored at 2-8 °C for one week or at -20 °C for nine months. Repeated freeze-thaw cycles should be avoided.
Reference values	Values are dependent on the type of sample collection procedure. Collagen IV (µg/g Creatinine): • Early morning urine: 30-39 years <4,0; > 40 years < 4,9. • Spot urine: >21 years < 7,3.

Urine sample collection tubes including urine stabilizing buffer to preserve urine proteins • TE1050 Box with 98 tubes – ready to use. • TE1055 Vial with 10 ml.	
Sample type	Urine.
Sample preparation	As soon as possible after sample collection, add 3.2 ml of urine to the collection tube provided, up to the 4.0 ml mark.

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