Summary Data Package

Nonclinical Enablement of Drug-Induced Skeletal Myopathy Translational Biomarkers

The Skeletal Muscle Working Group
of
Critical Path Institute's
Predictive Safety Testing Consortium

Table of Contents

List of Tables

List of Figures

List of Abbreviations

APAP	Acetaminophen
AST	Aspartate Transaminase
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
AUC	Area under the curve
CK	Creatine Kinase
Ckm	Creatine Kinase Muscle
C-Path	Critical Path Institute
cTnI	Cardiac troponin I
cTnT	Cardiac Troponin T
Fabp3	Fatty Acid Binding Protein 3
H&E	Hematoxylin-eosin
IDI	Integrated Discrimination Improvement
IP	One minus specificity
IS	Integrated sensitivity
LDH	Lactate dehydrogenase
LLOQ	Lower Limit of Quantification
Mb	Myoglobin
Myl3	Myosin Light Chain 3
NRI	Net Reclassification Improvement
PSTC	Predictive Safety Testing Consortium
PK	Pyruvate kinase
ROC	Receiver Operator Characteristics
SKM	Skeletal Muscle
SKMWG	Skeletal Muscle Working Group
TMPD	2,3,5,6-Tetramethyl-P-phenylenediamine
sTnI	Skeletal Troponin I

1. Executive Summary

This Biomarker Qualification Briefing Package reports the results of the Critical Path Institute's (C-Path) Predictive Safety Testing Consortium (PSTC) Skeletal Muscle Working Group's (SKMWG) analysis of several serum and plasma biomarkers of drug-induced skeletal muscle (SKM) degeneration/necrosis in rats and a chronic study in dogs. The results are derived from 34 toxicology studies in rat including 18 studies (one in aged rats) with nine compounds that cause degeneration/necrosis in SKM, and 16 studies with 14 compounds that induce degeneration/necrosis in other target tissues but not in SKM. Methods for assay validation, histopathology practices, and statistical analyses are included. This work has been conducted in support of the future clinical qualification of biomarkers of drug-induced SKM injury.

The data presented here evaluated four plasma/serum skeletal muscle biomarkers: Skeletal Troponin I (sTnI), Myosin Light Chain 3 (Myl3), Creatine Kinase M Isoform (Ckm), and Fatty Acid Binding Protein 3 (Fabp3). The comprehensive analyses of these data provide evidence to demonstrate that the novel SKM biomarkers sTnI, Myl3, Ckm, and Fabp3 outperformed aspartate transaminase (AST) and creatine kinase (CK) for diagnosis of drug-induced SKM injury in rats. In addition, when used in conjunction with CK and AST, these four biomarkers improved diagnostic sensitivity and specificity, as well as diagnostic certainty, for SKM injury and respond in a sensitive manner to low levels of SKM degeneration/necrosis in rats.

2. Background

Novel accessible biomarkers of drug-induced SKM injury, defined as degeneration/necrosis, are necessary to supplement the currently available biomarkers, serum AST and CK activities. Both AST and CK have been used for decades to help diagnose SKM injury in humans and animals and are key components of current clinical pathology panels. Advantages of CK and AST include the widespread availability of robust and low cost assays, and the extensive experience of toxicologists, researchers, clinicians, and regulatory scientists with these biomarkers. However, circulating CK and AST also have important technical and interpretive disadvantages including lack of tissue specificity for skeletal muscle, insufficient sensitivity for skeletal muscle necrosis in rats and in humans, and inability to use CK and AST to determine the predominant fiber type(s) of injured myocytes. Therefore, the individual companies participating in PSTC's SKMWG have pursued novel biomarkers of skeletal muscle degeneration/necrosis in their own laboratories. These novel biomarkers are meant to overcome the main disadvantages of CK and AST.

The biomarkers of skeletal muscle degeneration/necrosis evaluated in this qualification project include sTnI, Myl3, Ckm (protein assay), and Fabp3. Per convention in the literature for SKM, the descriptions below consider slow twitch muscle as Type I and fast twitch muscle as Type II.

2.1. Biomarkers of skeletal muscle degeneration/necrosis

2.1.1. Skeletal Troponin I (sTnI)

Troponin is a protein trimer consisting of three subunits: calcium-responsive (C), inhibitory (I), and tropomyosin-binding (T). Troponin regulates the interaction of myosin with actin necessary for the control of muscle contraction. There are multiple genes for each subunit and splice isoforms for some of the subunits. Expression of troponin subunit isoforms is specific to each muscle type (i.e. cardiac, slow-twitch skeletal muscle or fast-twitch skeletal muscle). For example, expression of troponin I, slow skeletal muscle is specific to slow twitch Type I skeletal muscle fibers. Whereas, troponin I, fast skeletal muscle is specific to Type II fast-twitch skeletal muscle fibers (Mullen 2000, Nikovits 1990). Cardiac troponin I (cTnI) and T (cTnT) are expressed in cardiac muscle and are not expressed in skeletal muscle (McLaurin 1997). For this qualification document, we use the abbreviation sTnI to refer to both the fast and slow skeletal muscle troponin I proteins. sTnI is anticipated to increase sensitivity and especially specificity when compared to established biomarkers of SKM injury.

2.1.2. Creatine Kinase M (Ckm)

The cytosolic form of the creatine kinase (CK) enzyme is responsible for the regeneration of adenosine triphosphate (ATP) from phosphocreatine and adenosine diphosphate (ADP) (Wallimann 1992). Phosphocreatine is the high energy intermediate used to rapidly transfer energy from mitochondrial ATP to cytosolic ATP for the contraction of cardiac and skeletal muscle. Cytosolic CK is a dimeric protein with subunits coded by the CKM (muscle) and CKB (brain) genes. The subunits are differentially expressed, giving rise to three cytosolic CK isoforms: MM, BB and MB. In skeletal muscle 90% of CK is the CK-MM homodimer (Ckm), whereas in heart CK consists mostly of CK-MB and to a lesser extent CK-MM (Apple 1984). While measuring CK activity does not discriminate between isoforms, measuring Ckm protein (i.e. CK-MM homodimer) is highly selective for skeletal muscle CK. Ckm, as part of a panel of SKM and cardiac toxicity markers, may increase sensitivity and specificity when compared to established biomarkers of SKM injury.

2.1.3. Myosin Light Chain 3 (Myl3)

Myl3 is an essential light chain of the myosin molecule expressed predominantly in cardiac and skeletal muscle. Myosin is a six subunit mechano-chemical enzyme that functions to couple the hydrolysis of ATP to conformational changes that result in movement of the protein complex along actin filaments. This hexameric protein consists of two heavy chain subunits, which form the head and tail domains of the complex. The heavy chains are held together by four light chain subunits. The light chain subunits consist of two regulatory light chains with phosphorylation sites (encoded by the MYL2 genes), and two essential light chains (encoded by the MYL3 genes). Following damage to muscle tissue, the constituent subunits of myosin become dissociated, and Myl3 is released into the blood stream. To date, efforts have focused on determining the utility of Myl3 as a biomarker of cardiomyocyte injury (Berna 2007, Lee 2005). Given its abundant expression in type I skeletal muscle, it has been noted that Myl3 may also be

useful as a circulating surrogate for injury to this tissue type (Berna 2007). Tonomura et al, (2012) investigated the change in circulating Myl3 in response to a panel of cardiac and skeletal muscle toxicants. Myl3 accurately detected injury, but could not differentiate between skeletal muscle and cardiac muscle toxicity. In the same study rats treated with a panel of hepatobiliary toxicants had no measureable increase in plasma Myl3. Because Myl3 is found in both cardiac and SKM fibers, increases of Myl3 should be interpreted in conjunction with histopathology or other specific diagnostic tests.

2.1.4. Fatty Acid Binding Protein 3 (Fabp3)

Fabp3 (also known as heart-type cytoplasmic fatty acid binding protein or H-Fabp) is a small (14.5 kDa) cytoplasmic protein that plays a permissive role in transport/mobilization of fatty acids within the cellular environment. Fabp3 binds both saturated and polyunsaturated fatty acids with high affinity (Kd 2-60nM). It is thought to shuttle fatty acids from the plasma membrane to intracellular sites of usage including the β-oxidation machinery. Fabp3 has also been shown to be expressed in SKM and increases in response to physiological conditions that increase fatty acid demand/availability, such as testosterone, endurance training, and nutritional state (Glatz 2003). Therefore, Fabp3 has been proposed as a biomarker of both cardiac and SKM damage.

Several studies have linked Fabp3 with SKM toxicity. Circulating levels of Fabp3 correlated well with the incidence and severity of SKM necrosis. When compared with existing biomarkers of skeletal muscle injury, Fabp3 was the most robust in concordance, sensitivity, positive and negative predictivity, and false negative rate (Pritt 2008). A direct correlation between circulating concentrations of Fabp3 and PPAR-α-agonist-induced type I muscle fiber toxicity was observed in rats (Pritt 2008). This study also demonstrated focal depletion of Fabp3 from injured muscle fibers. In a study in rats treated with the acetylcholinesterase inhibitor carbofuran (CAF), skeletal myotoxicity was observed histopathologically and correlated well with plasma levels of Fabp3 (Tonomura 2009). In another rat study testing a panel of myotoxicants Fabp3 was found to be a sensitive marker for both cardiac and musculoskeletal toxicity. However, Fabp3 was not able to differentiate between these two types of injury (Tonomura 2012). Rats treated with hepatobiliary toxicants showed no increase in Fabp3. Because Fabp3 is found in both cardiac and SKM fibers, increases of Fabp3 should be interpreted in conjunction with histopathology or other specific diagnostic tests.

2.2. Biomarker tissue concentration and specificity

To confirm literature reports of biomarker specificity for SKM tissue in rats, rat tissue lysates were generated from selected tissue samples from three naïve female and three naïve male Sprague Dawley rats. The protein levels in the lysates were then determined, and these individual animal lysates used to create protein mass-balanced pools at a concentration of 5 mg/mL. The pools were then diluted to fall within the quantifiable range of the SKM biomarker assays, and the estimated concentration adjusted for the dilutions of each biomarker. The results are presented in Table 1 and expressed as ng of analyte per mg of tissue based on the calibrator in the respective assay. These data illustrate the SKM specificity for the proposed novel biomarkers, supporting increased presence in the circulation (serum or plasma) as a marker of

SKM damage. The Ckm assay results comparing the amount detected in skeletal muscle tissue versus heart tissue support the claim of preferential detection of the creatine kinase M-type protein. However, we cannot, at this point, exclude the possibility of the potential binding of the creatine kinase B-type protein due to the very similar amino acid sequence of the two proteins. The concentrations reported for Ckm are based on calculations with the assay calibrator that appears to overestimate protein. Accordingly, Ckm concentrations should be considered relative (i.e. 100-fold greater amount of Ckm in skeletal muscle tissue).

Table 1 Estimated Tissue Concentrations of Candidate Biomarkers in Sprague Dawley Rat Tissue Lysates

	Analyte Concentrations (ng/mg tissue)				
Tissue	**sTnI**	**Fabp3**	**Myl3**	**Ckm**	**cTnI**
SKM: Quadriceps (minus VI)	4200	2100	27	2700000	0
SKM: Soleus	730	12000	2500	1100000	0
SKM: Vastus Intermedius	3100	7800	890	1700000	0
SKM: Biceps	3000	2400	110	2300000	0
SKM: Extensor Digitorum Longus	3300	2300	74	2300000	0
SKM: Gastrocnemius	3500	2400	170	2500000	0
Heart	8	11000	500	21000	17000
Kidney	2	763	0	31	0
Liver	2	6	0	580	0
Bladder	0	22	0	430	0
Brain	2	238	0	730	0
Duodenum	0	9	0	44	0

2.3. Rationale and Supporting Evidence for Clinical Translation

The diagnosis and monitoring of skeletal muscle injury in the clinical setting poses many of the same challenges as in the pre-clinical drug development setting. In the clinic, this issue is further complicated because muscle biopsy for direct microscopic evaluation is often not feasible (Clarkson 2002, Goodwin 2011). Other non-invasive methods, such as magnetic resonance imaging (MRI), are available to image muscle tissue but are often difficult to interpret or impractical to use routinely in a clinical trial (Goodwin 2011). In addition, as noted previously for the rodent, the conventional clinical chemistry biomarkers lack specificity for skeletal muscle tissue (Brancaccio 2011, Wu 1992, Bagley 2007). Therefore, the identification of translatable circulating biomarkers with increased sensitivity and specificity for skeletal muscle injury would significantly benefit drug development. The proteins proposed here (Ckm, Myl3, sTnI and Fabp3) for endorsement as biomarkers of skeletal muscle degeneration/necrosis in pre-clinical

drug safety studies in the rat are highly homologous to the human proteins. An alignment of the National Center for Biotechnology Information (NCBI) reference protein sequences showed that rat sTnI shares 96% amino acid identity with human sTnI. Rat Ckm, Fabp3 and Myl3 amino acid sequences are 96%, 89% and 87% identical, respectively, to the human proteins. Furthermore, the proteins display a very similar tissue distribution in humans and serve the same role in skeletal muscle biology as they do in the rat (Schiaffino 2011). This suggests the preclinical biomarkers proposed here may translate to the clinic. The subsequent paragraphs summarize the clinical literature on circulating biomarkers for monitoring muscle injury and the evidence supporting the biomarkers proposed here.

Typical clinical signs of severe skeletal muscle injury (rhabdomyolysis) include muscle pain, weakness and brown urine (Bagley 2007). Subsequent serious complications resulting from rhabdomyolysis include acute renal failure (Bagley 2007, Abassi 1998, Singh 2005), which may occur with drug-induced myopathies (Kuncl 2009, Owczarek 2005). More sensitive and specific biomarkers would aid the diagnosis of drug-induced skeletal muscle degeneration/necrosis and potentially aid in discrimination of injuries to skeletal versus cardiac muscle. Currently, the most commonly used serum biomarkers (see next paragraph) cannot discriminate between skeletal and cardiac muscle injury (Wu 1992, Matsumura 2007, Van Hoof 2012).

Historically, numerous circulating biomarkers have been used in the clinic for the diagnosis of skeletal muscle injury including CK, AST, lactate dehydrogenase (LDH), pyruvate kinase (PK), and myoglobin (Mb) (Brancaccio 2010, Wu 1992, Bagley 2007). All of these markers suffer from a lack of specificity for skeletal muscle tissue (Brancaccio 2010, Baird 2012). Of these, the CK enzymatic assay is the most commonly used clinical measure of skeletal muscle injury in humans and has been used extensively in drug safety evaluation (Kuncl 2009, Owczarek 2005, Lee 2005, Shepherd 2004), muscle disease research (Okinaka 1961, Zatz 1991), surgical medicine (Cohen 2009, Lambert 2002), sports medicine (Baird 2012, Banfi 2012, Coutts 2007, Kamandulis 2011, Paul 1989, Brancaccio 2007), and nutrition research (Bassit 2010, Cockburn 2010, Kon 2008, Rawson 2007, Sacheck 2003, Hoffman 1996). Though much less commonly used, the clinical application of isoenzyme specific CK mass assays has been reported for the monitoring of muscle disease progression and in sports medicine (Brancaccio 2007, Hoffman 1996, Leiserowitz 1992, Mokuno 1987, Noakes 1983). There are, however, limitations to the utility of enzymatic activity of circulating CK as a biomarker. For example, additional analysis of CK-MB or cardiac troponins is required to distinguish SKM damage from cardiac injury. In addition, in pathophysiological states involving glutathione depletion, there is a significant reduction in CK enzyme activity which affects assay performance (Gunst 1998). CK-MM has been shown by isoelectric focus electrophoresis and immunoassay to be the predominant CK isoform released in response to SKM injury caused by eccentric exercise (Apple 1988, Lo 2008). However, the evaluation of Ckm, as a specific marker of SKM injury has not been reported.

Citing the successful application of cardiac troponin assays for monitoring cardiac tissue injury (Matsumura 2007, Jaffe 2012), numerous clinical studies have investigated the utility of sTnI as a specific biomarker of SKM injury. Immunoassays using isoform specific antibodies for human sTnI have been developed and used to accurately measure sTnI concentrations in clinical serum samples (Takahashi 1996). Measurement of circulating concentrations of sTnI was applied to the clinical study of skeletal muscle disease (Takahashi 1996, Simpson 2005, Simpson 2002) and

exercise and trauma-induced muscle damage (Chapman 2013, Foster 2012, Matziolis 2011, Sorichter 1997). Several investigators have reported sTnI as a more specific biomarker of skeletal muscle injury than conventional biochemical markers (Takahashi 1996, Foster 2012).

Circulating levels of Fabp3 have been studied as a potential clinical biomarker of both cardiac and skeletal muscle damage (Pelsers 2005); however, much of the work has focused on its value in the diagnosis of early myocardial damage (Dellas 2010, Gururajan 2010, Inoue 2011, Malik 2006, McCann 2008, Muehlschlegel 2010, Niizeki 2007). Sorichter, et al., investigated circulating Fabp3 and Mb as early markers of exercise-induced skeletal muscle damage in healthy human volunteers. They reported that the ratio of Fabp3 to myoglobin concentrations was a more sensitive and specific assay for early detection of skeletal muscle injury than CK (Sorichter 1998). Several investigators have recommended the use of Fabp3 as part of a multi-marker approach to increase the prognostic value of individual tissue injury biomarkers (Sorichter 1998, McCann 2009, Xu 2010).

Two recent reports have utilized proteomics approaches to identify circulating biomarkers of skeletal muscle injury in muscular dystrophy patients. Ayoglu, et al used an antibody-mediated affinity approach and identified Ckm and Myl3, along with seven proteins involved in striated muscle function and metabolism, as significantly elevated in the blood of muscular dystrophy patients (Ayoglu 2014). Hathout et al. employed an *in vivo* labeling approach to compare the serum proteomes of the mdx mouse model of muscular dystrophy to wild-type controls. They identified 23 significantly elevated proteins in the serum of mdx mice, including Ckm, Myl1/3 and Fabp3 (Hathout 2014). The authors then used a mass spectrometry approach to confirm that these proteins were also significantly elevated in the serum of Duchenne muscular dystrophy patients. These recent publications provide strong evidence, using multiple analytical approaches, for the likely clinical translatability and utility of these skeletal muscle injury biomarkers.

2.4. Model compound and dose selection

Compounds chosen as models of drug-induced SKM degeneration/necrosis in the rat were selected from well-characterized toxicants described in the literature. They include cerivastatin, PPAR-alpha agonists and 2,3,5,6-Tetramethyl-P-phenylenediamine (TMPD), among others. In addition to these SKM toxicants, member participants contributed several proprietary developmental compounds that exhibited SKM degeneration/necrosis in nonclinical safety assessment studies. These novel compounds further diversify the chemical space tested beyond the published literature. Compounds and study designs are summarized in Table 2. In addition, to investigate the response of our proposed biomarkers during the resolution phase of SKM injury, a subset of these studies included a recovery arm.

To investigate the specificity of our proposed biomarkers for monitoring SKM degeneration/necrosis, a series of toxicity studies with compounds causing degeneration/necrosis in target organs other than SKM were also conducted. Collectively, the studies causing SKM degeneration/necrosis are referred to as “sensitivity” studies, and the studies causing injury to other tissues, but not skeletal muscle, are referred to as “specificity” studies.

Furthermore, we conducted a SKM toxicology study in aged rats to examine the impact of age on baseline biomarker values and responsiveness to SKM injury. Potential effects of age, as well as other factors such as impact of exercise, are an important consideration for future clinical use of the SKM biomarkers.

3. Methods

3.1. Studies

Rat toxicology studies were designed to evaluate the performance of the skeletal muscle biomarkers in comparison to CK and AST as benchmarked against myocyte injury observed via histopathology. A total of 34 rat toxicity studies were conducted, consisting of 17 sensitivity studies (inducing SKM degeneration/necrosis), 16 specificity studies (inducing injury in tissues other than skeletal muscle) and one study in one year old rats. Seven of the sensitivity studies included a recovery arm to evaluate response of biomarkers to resolution of SKM injury. The final dose regimens were determined from the literature and/or dose range finding studies. In total, the compound set used in these studies provided a variety of pathogenic avenues that culminated in skeletal myocyte degeneration/necrosis, as well as a diverse set of additional toxicological outcomes that might be encountered in drug development.

1 **Table 2 Summary of study designs for all rat studies**

Study Number	Compound	Model; target organ toxicity	Dose(s) (mg/kg)	Frequency (# of doses)	Necropsy Day(s)	Route	Dose Group Size	Sex	Strain	Recovery+
1143	BMS Compound	Sensitivity; SKM	0, 50, 100	Daily (3 doses)	4	PO	6	M	SD	Yes
1144	Cerivastatin	Sensitivity; SKM	0, 1	Daily (1, 6, 8, 10, or 14 doses)	2, 7, 9, 11, or 15	PO	8/TP	F	SD	No
2080	Cerivastatin	Sensitivity; SKM	0, 0.03, 0.1, 0.3, 0.6, 1	Daily (6 or 13 doses)	7 or 14	PO	6/TP	F	SD	No
2081	Cerivastatin	Sensitivity; SKM	0, 0.03, 0.3, 3	Daily (6 or 13 doses)	7 or 14	PO	6/TP	F	SD	No
2089	Cerivastatin	Sensitivity; SKM	0, 0.6	Daily (10 doses)	11	PO	5	F	SD	Yes
2772	Cerivastatin	Sensitivity; SKM	0, 0.5, 1	Daily (14 doses)	15	PO	5	F	Wistar	No
2776	Cerivastatin	Sensitivity; SKM	0, 0.71, 1.42	Daily (4, 9, or 14 doses)	5, 10, or 15	PO	5/TP	F	SD	Yes
2079	Lilly GPCR	Sensitivity; SKM	0, 100, 125	Daily (10 or 14 doses)	11 or 15	PO	6	F	Fischer	No
2082	Lilly PPARα agonist	Sensitivity; SKM	0, 100, 300, 1000	Daily (4 doses)	5	PO	6/TP	F	SD	No
2089	Lilly PPARα agonist	Sensitivity; SKM	0, 300	Daily (5 doses)	6	PO	5	F	SD	Yes

Study Number	Compound	Model; target organ toxicity	Dose(s) (mg/kg)	Frequency (# of doses)	Necropsy Day(s)	Route	Dose Group Size	Sex	Strain	Recovery+
2078	Lilly Re-Uptake Inhibitor	Sensitivity; SKM	0, 35, 50	Daily (10 or 14 doses)	11 or 15	PO	6/TP	F	Fischer	No
2765*	Merck B	Sensitivity; SKM	0, 50, 100, 250	Daily (3 doses)	5 or 9	PO	4	F	SD	No
2200	Pfizer Compound	Sensitivity; SKM	0, 5, 50, 100	Daily (14 doses)	15	PO	10 (15 veh.)	M	Wistar	Yes
2775	PPARα agonist	Sensitivity; SKM	0, 5, 25, 150	Daily (7 doses)	8	PO	5	F	SD	Yes
2088	TMPD	Sensitivity; SKM	0, 0.3, 0.6, 1, 3, 6, 9	Single	2	PO	6	F	SD	No
2089	TMPD	Sensitivity; SKM	0, 9	Single	2	PO	5	F	SD	Yes
2766	TMPD	Sensitivity; SKM	0, 2, 4, 6	Daily (1, 2, or 7 doses)	1, 3, 8	SQ	5/TP	M	SD	No
3484†	TMPD	Sensitivity, SKM; aged animals	0, 0.1, 0.5	Single	2	PO	5	M	SD	Yes
2764‡	Merck C	Specificity; GI	0, 100, 300, 900	Daily (1 or 7 doses)	2 or 8	PO	4	F	SD	No
2774	Merck D	Specificity; GI	0, 100, 500	Daily (4 or 5 doses)	6	PO	5	F	SD	No
3046‡	Merck E	Specificity; GI	0, 30, 100	Daily (7 doses)	8	PO	5	F	SD	No

Study Number	Compound	Model; target organ toxicity	Dose(s) (mg/kg)	Frequency (# of doses)	Necropsy Day(s)	Route	Dose Group Size	Sex	Strain	Recovery+
1034‡	Cisplatin	Specificity; kidney	0, 1.5	Daily (1, 3, or 7)	2, 4, or 8	IV	5/TP	F	SD	No
2770*‡	Cisplatin	Specificity; kidney	0, 0.5, 3.5, 7	Single	4 or 8	IV-1mL/min	4/TP	M	Wistar	No
2777*‡	Doxorubicin	Specificity; kidney	0, 4, 8	Daily (1, 8, or 15 doses)	14, 28, 42	IV-bolus dose	8/TP	M	Wistar	Yes
1034‡	Lilly - Enzyme Inhibitor	Specificity; kidney	0, 1200	Daily (1, 3, or 7 doses)	2, 4, or 8	PO	5/TP	F	SD	No
2769*	Propylenamine	Specificity; kidney	0, 11, 22	Single	7 or 21	IP	5/TP	M	Wistar	No
2086*	Thioacetamide	Specificity; kidney, liver	0, 30, 100	Single	1, 4, or 5	IP	5/TP	M	SD	No
2767*	Thioacetamide	Specificity; kidney, liver	0, 50, 100, 200	Single	2 or 3	PO	5/TP	M	SD	No
2084*	α-naphthyl isothiocyanate	Specificity; liver	0, 30, 100	Single	1, 2, 3, 4, 5, or 6	PO	6/TP	M	SD	No
3484†	Acetaminophen	Specificity, aged animals; liver	0, 1400	Single	2	PO	5	M	SD	Yes
2768	Bromobenzene	Specificity; liver	0, 75, 300, 750	Single	2	IP	4	M	SD	No
3048	Furan	Specificity; liver	0, 4, 40, 60	Daily (1 or 3 doses)	2 or 4	PO	5/TP	M	SD	No

Study Number	Compound	Model; target organ toxicity	Dose(s) (mg/kg)	Frequency (# of doses)	Necropsy Day(s)	Route	Dose Group Size	Sex	Strain	Recovery+
2087*	Methyl-enedianiline*	Specificity; liver	0, 25, 50, 75, 100	Single	1, 2, 3, 4, or 5	PO	3/TP	M	SD	No
2778	Angiotensin II	Specificity; vasculature	0, 341, 383, 949, 984	Daily (14 or 28 doses)	14 or 28	SQ-Osmotic Pump	8/TP	M	SD	No

***** Denotes studies in which a necropsy occurred greater than 24 hours following last dose
† Study 3484 was analyzed separately from the other biomarker studies. Data were used only to look at SKM biomarkers in aged rats.
‡ These studies were not included in ROC or IDI/NRI analyses due to absence of CK data.
+ Study design of recovery arms are not indicated in this table as this data was analyzed separately.

3.2. Cross-site assay validation

The SKMWG conducted a multi-site characterization and validation of the technical performance of assays for Fabp3, Myl3, sTnI, and Ckm. All sites contributing data to the biomarker qualification dataset participated in the validation exercise. The assays selected for biomarker measurement were MesoScale Discovery's Muscle Injury Panel 1 (containing assays for sTnI, Myl3, and Fabp3) and Muscle Injury Panel 2 (including Ckm). MesoScale Discovery assay technology uses a chemiluminescent ELISA antibody platform.

Assay validation activities were informed by relevant guidances (FDA 2001, EMA 2012). Serum and/or plasma samples from rats treated with SKM toxicants were aliquoted and shipped to each participating site for use as high, mid and low analyte concentration quality control samples. Calibrators provided by the manufacturer were also included. According to the manufacturer's validation reports, the SKMWG established target acceptance criteria for intra-assay and inter-assay precision, limits of quantitation, sample linearity, matrix/recovery, and stability (freeze/thaw and storage).

3.3. Histopathology methods

3.3.1. Overview

Pathologists from participating institutions developed recommendations for the histopathologic assessment for novel SKM biomarker studies. In summary, the soleus muscle and the quadriceps muscle group were collected as standard specimens with additional muscle samples included in some studies. The quadriceps group is comprised of 5 individual muscles including Type I-rich (vastus intermedius) and Type II-rich (tensor fascia lata, rectus femoris, vastus medialis, and vastus lateralis) muscles. Therefore, including the Type I-rich soleus muscle, up to 6 muscles were evaluated. Non-skeletal muscle tissues routinely collected for the SKMWG biomarker qualification studies included heart (including aorta), liver, and kidney. Standard collection, handling, trimming, and processing methods were utilized for production of hematoxylin-eosin (H&E) stained microscopic slides.

To foster consistency of evaluation between contributing institutions and to enable statistical evaluation of the data for biomarker qualification, a lexicon was adopted for classification of microscopic changes in skeletal muscle. Additionally, guidelines were described for a five-point (Grades 0 to 4) severity scoring system for microscopic changes. The primary and peer review histopathology evaluations were conducted by qualified veterinary pathologists according to procedures recommended for nonclinical safety biomarker qualification studies (Burkhardt 2011). Approaches for diagnostic thresholding and for recording features considered to represent handling artifact or common background/spontaneous changes were also prescribed.

3.3.2. Histopathological evaluation and binning of diagnoses

Although the lexicon was sufficiently sophisticated to capture multiple types of skeletal muscle injury, we specifically queried whether our biomarkers of interest could diagnose myocyte degeneration/necrosis. The four proteins, Myl3, Ckm, Fabp3, and sTnI, would be expected to be

released into circulation upon rupture or leakage of the sarcolemma, regardless of the pathogenesis leading to the membrane injury. For the purposes of the statistical analysis, skeletal muscle injury was defined as "degeneration/necrosis, myocyte" and for each animal the highest histopathology severity score recorded for any examined muscle sample was used for the analysis. The endpoints of degeneration and necrosis are combined to a single endpoint, because they are often concurrent, the myofiber size may preclude evaluation along its entire length, and the infrequent occurrence of degeneration alone will be captured by other morphologic endpoints in the lexicon (i.e. vacuolation and hyaline changes).

Because AST is also expressed in liver tissue and AST, CK, Myl3, Ckm, and Fabp3 are also expressed in cardiac tissue, some data analyses examined biomarker behaviors when liver or cardiac injury were present. Because not all diagnoses recorded for liver or heart would be expected to be associated with the potential release of intracellular biomarkers, an effort was made to bin these diagnoses according to perceived relevance. A board-certified pathologist reviewed and curated the data recorded for each animal with liver or cardiac diagnoses according to whether each diagnosis was likely or unlikely to have resulted in biomarker release from that tissue. Examples of liver changes classified as not likely associated with biomarker release are increased hepatocellular mitotic figures and decreased hepatocellular glycogen. Examples of liver changes classified as likely associated with biomarker release from liver cells are multifocal hepatocellular necrosis and hepatocellular single cell necrosis.

3.4. Data analysis

3.4.1. Overview

The statistical data analysis for this project was designed to address three primary objectives as follows:

Primary objective 1: Characterize the performance of individual novel SKM biomarkers relative to the standard biomarkers AST and CK. This comparison was made using receiver operator characteristics (ROC) analysis, which is a commonly accepted method for evaluating the overall sensitivity and specificity of an index.

Primary objective 2: Determine if the combination of each novel biomarker with AST and CK significantly improves performance as measured by ROC area under the curve (AUC). A Net Reclassification Improvement (NRI) and Integrated Discrimination Improvement (IDI) analysis was also used to determine if adding each novel biomarker to AST and CK could improve diagnostic certainty.

Primary objective 3: Examine the ability and specificity of individual novel biomarkers to detect SKM injury when cardiac or liver injury are also present.

Skeletal muscle injury for all analyses was defined as the histopathology lexicon term "degeneration/necrosis, myocyte." This common microscopic endpoint, regardless of inciting cause or pathogenic mechanism, clearly reflects situations of compromised myocyte integrity allowing release of intramyocellular proteins, such as the proposed new biomarkers, into the blood. Other terms from the histopathology lexicon that might involve compromised membrane

integrity, such as "vacuolation, sarcoplasm", could be considered for similar future statistical analyses but were not included in the statistical analyses at this time.

3.4.2. Analysis setup

3.4.2.1. Pre-processing biomarker measurements

Biomarker measurements were analyzed on the log scale (base 2). Plots of the log scale data did not suggest excessive skewing. Marker values analyzed on the log scale were normalized to concurrent control by subtracting the mean of controls from each value. For this purpose, marker values were matched with a control mean calculated from control rats of the same gender, in the same study and measured on the same day. Biomarker values below the lower limit of quantification (LLOQ) were replaced by the value LLOQ/2.

3.4.2.2. Defining groups for analysis

The ROC and logistic regression analyses involved the comparison of a positive to a non-positive group of samples. Group membership was based on the skeletal muscle histomorphological change composite scores in an inclusion analysis as described below. The composite score was assigned as the highest severity skeletal muscle diagnosis for “degeneration/necrosis, myocyte” for any muscle tissue recorded.

3.4.2.2.1. Inclusion analysis

Inclusion analysis makes no provision for the possibility of errors in the histomorphological change composite scores. Samples from animals without a positive score are treated as non-positives regardless of their treatment regimen. Likewise, samples from animals with a positive score are treated as positives regardless of whether their treatment regimen was control or a compound not expected to be toxic to skeletal muscle tissue.

3.4.3. Evaluation of diagnostic performance

Univariate marker performance was measured using the muscle histomorphological change scores described above. The scores were dichotomized by assigning a value of 0 to those animals with no injury and 1 to animals with myocyte degeneration/necrosis. This variable was used in a binary logistic regression model to predict the possibility of skeletal muscle degeneration/necrosis based on a given biomarker concentration. The output of this model was used to provide estimates of:

1. AUC from a ROC curve.
2. Differences in AUC between biomarkers or models.
   AUC of the biomarkers was compared using a contrast statement with the Wald Chi-square test statistic as provided by the ROC contrast statement in proc logistic (SAS v. 9.3).
3. Sensitivity for a pre-selected (95%) specificity level.

3.4.4. Evaluation of improved diagnostic certainty

Assessment of the amount of information each biomarker adds to the information already contained in the model with the two standard markers (AST and CK) was accomplished by fitting nested logistic regression models. The basic procedure was to compare the performance statistics of the model containing the novel SKM biomarker with the standard markers to the performance statistics of the model containing only the standard markers.

NRI is based on the proportion of individuals for which addition of the marker improves the performance of the predicted diagnosis. In other words, NRI moves the predicted value of animals with positive histomorphological change scores towards 1 or shifts the predicted value of animals with non-positive histomorphological change scores towards zero. NRI was evaluated with the goal of providing a more easily interpreted metric. The NRI was calculated from the nested binary logistic regression models. The fraction improved for all findings was calculated by averaging the fraction improved for positive findings and the fraction improved for negative findings.

IDI was performed as a companion analysis to combine measures of integrated sensitivity (IS) and "one minus specificity" (IP) into a single metric for testing. IS was estimated by the mean predicted value for the samples with positive skeletal muscle histopathological change scores and IP was estimated by the mean predicted value for the samples with non-positive skeletal muscle histomorphological change scores. Estimates of IS and IP parameters were calculated using binary logistic regression models. If the IS is the mean diagnostic value for positive samples, integrated IP is the mean diagnostic value for negative samples and we consider the model with the biomarker as the "new" model and the model without the biomarker the "old" model, then $IDI = [IS_{new} - IS_{old} - (IP_{new} - IP_{old}) = (IS_{new} - IS_{old}) + (IP_{old} - IP_{new})]$ which equals the sum of the mean improvement for positives and the mean improvement for negatives. Then IDI is a composite indicator of overall improvement of each candidate biomarker's diagnostic certainty. Larger IS and smaller IP indicate better markers.

3.4.5. Assessment of biomarker change with injury severity

The ROC, IDI, and NRI analysis rely upon a binary classification of animals as either injured or not injured. Of interest was whether individual biomarker values correlate with gradations of SKM degeneration/necrosis as assessed by a histopathology severity score (0-4). A simple plot of the biomarker change over concurrent within study control for histopathology severity score was drawn; a $\log_2$ transformation was performed for display purposes. A Spearman correlation coefficient test was performed to determine the significance of the relationship between each biomarker and the histopathology score.

3.4.6. Assessment of biomarker preferential specificity for SKM over liver or cardiac tissue

To examine the behavior of individual SKM biomarkers when liver or cardiac injury was present, a board-certified pathologist reviewed animals with liver and cardiac injury pathological diagnoses. Some animals also had concurrent SKM injury and some did not. Simple plots of

biomarker fold-changes over concurrent control were made to compare biomarker levels in animals with no injury, cardiac injury only, SKM injury only, and SKM + cardiac injury.

3.4.7. Additional analyses in support of biomarker qualification

Several additional studies and analyses were performed in support of understanding biomarker behavior and toxicological utility under certain conditions. These include biomarker changes in animals recovering from drug-induced SKM injury, biomarker baseline levels and response to toxicological insult in aged animals, and a preliminary assessment of SKM biomarkers in canines (shown below).

4. Summary Results

Based upon the methods described above, the results of these studies demonstrate that serum Fabp3, Ckm, and Myl3 outperform AST and CK in identifying cases of skeletal muscle degeneration/necrosis; sTnI outperformed CK only. When used individually in combination with AST and CK, all four biomarkers improve the overall ability and sensitivity at 95% specificity to diagnose skeletal muscle degeneration/necrosis as compared to AST and CK alone. When used with AST and CK, all four biomarkers improve diagnostic certainty of correctly identifying cases of skeletal muscle degeneration/necrosis (Table 3). Each of the novel biomarkers sensitively signal very low levels of skeletal myocyte injury and increasing levels of the biomarkers correlate with increasing histopathologic severity scores for myocyte degeneration/necrosis. Finally, following drug-induced SKM injury, biomarker levels decrease with resolution of injury as observed via histopathology during a period of recovery.

Table 3 Overall summary of results

Biomarker	Improves overall sensitivity and specificity when used with AST and CK	Greater sensitivity than AST and CK at 95% specificity	Greater sensitivity and specificity than AST and CK	Improves diagnostic certainty when combined with AST and CK	Levels in blood correspond to severity of SKM injury
Ckm	✓	✓	✓	✓	✓
Fabp3	✓	✓	✓	✓	✓
Myl3	✓	✓	✓	✓	✓
sTnI	✓	✓	CK only	✓	✓

4.1. ROC curve results

4.1.1. Novel SKM biomarker vs. AST or CK

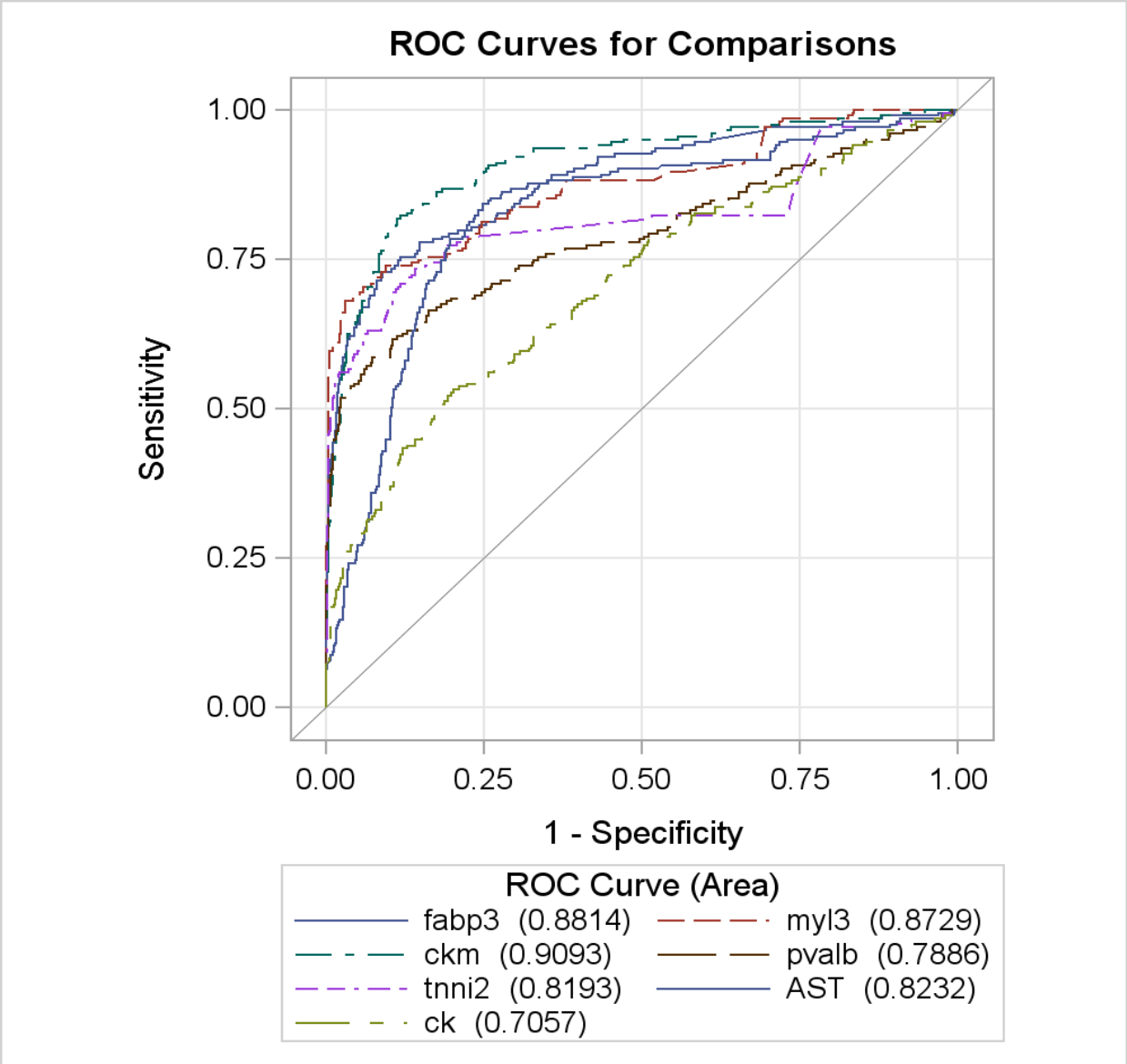
When comparing the AUC of the ROC curve of the individual novel biomarkers directly against AST or CK, the data demonstrate that Fabp3, Ckm, and Myl3 outperform AST and CK in identifying cases of skeletal muscle degeneration/necrosis (Table 4; Figure 1). The AUC for Ckm, Fabp3, and Myl3 is significantly greater than the AUC for AST. The AUC for Ckm, Fabp3, Myl3, and sTnI is significantly greater than the AUC for CK. AST and CK have poor sensitivity (26% and 28%, respectively) for drug-induced skeletal muscle injury at 95% specificity. The novel biomarkers under investigation also improved upon sensitivity. Fabp3 and Myl3 have 64% and 69% sensitivity respectively at 95% specificity.

Table 4 ROC curve results for novel SKM biomarkers compared to AST and CK

Biomarker	AUC	Prob AUC □ AUC_{AST}	Prob AUC > AUC_{CK}	Sensitivity at 95% Specificity
Ckm	0.9093	<0.0001	<0.0001	65%
Fabp3	0.8814	<0.0001	<0.0001	64%
Myl3	0.8729	<0.0116	<0.0001	69%
AST	0.8232	NA	<0.0001	26%
sTnI	0.8193	0.8628	<0.0001	59%
CK	0.7057	<0.001	NA	28%

p values in red indicate AUC significantly greater than comparator AUC. NA = not applicable

Figure 1 ROC curves for each of the novel SKM biomarkers and AST and CK.



4.1.2. Novel SKM biomarker + AST and CK vs. AST and CK

Our first ROC curve analysis (Figure 1) demonstrated that individually Ckm, Fabp3, and Myl3 each outperformed AST, and all individual biomarkers outperformed CK. As these novel biomarkers are proposed for voluntary use in addition to AST and CK, our next ROC analysis asked what the added benefit of using each individual new biomarker in conjunction with the traditional biomarker(s) would be.

Using an individual novel biomarker paired with both AST and CK only very slightly improved results for biomarkers that already significantly outperformed AST and CK as standalone indicators (i.e. Ckm, Myl3, and Fabp3). However, the AUC for sTnI plus AST and CK is significantly greater than that for AST and CK alone, with markedly improved sensitivity. ROC curves are shown for each biomarker plus AST and CK in Figure 2.

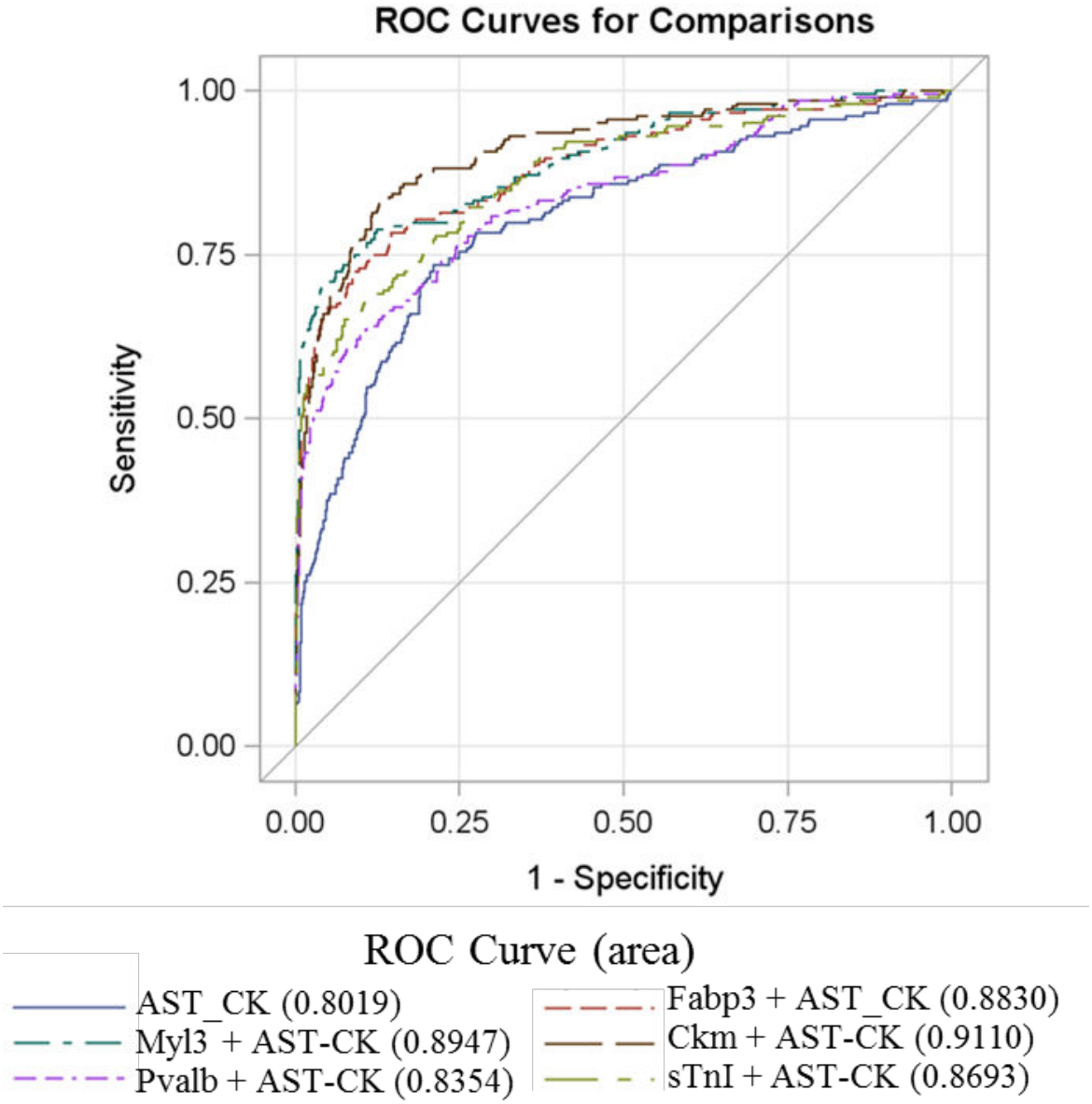
Sensitivity of the biomarkers at 95% specificity remained mostly unchanged in a ROC analysis for the biomarker used alone (Table 4), or in combination with AST and CK (Table 5; Figure 2).

Table 5 ROC curve results for novel SKM biomarker + AST and CK compared to AST and CK

AST and CK plus	AUC	Prob AUC □AUC$_{CK\ and\ AST}$	Sensitivity at 95% Specificity
Ckm	0.911	<0.0001	66%
Myl3	0.8947	<0.0001	71%
Fabp3	0.883	<0.0001	67%
sTnI	0.8693	<0.0001	58%
AST_CK	0.8019	NA	37%

Red indicates that the AUC from the model with a combination of AST, CK and the indicated biomarker is significantly greater than the AUC from the model with AST and CK alone, $p<0.05$, one tailed Wald's Chi Square test.

Figure 2 ROC curves for novel SKM biomarkers + AST and CK vs. AST and CK



4.2. NRI and IDI results

We also evaluated whether individual candidate biomarkers can improve the certainty of a diagnosis of skeletal muscle degeneration/necrosis. The criteria we used is based on significantly improving identification of both positive and negative cases of skeletal muscle injury. We defined significance as simultaneously having p-values less than $1x10^{-4}$ for:

- Improved detection of positive findings
- Improved detection of negative findings
- Total IDI

Based on these criteria we found that Fabp3, Ckm, Myl3, and sTnI individually are capable of improving the certainty of identifying both positive and negative cases of drug-induced skeletal muscle degeneration/necrosis when used with AST and CK, when compared to AST and CK alone (Table 6). Thus, each of the individual novel biomarkers significantly increase predictive certainty in identifying drug-induced skeletal muscle degeneration/necrosis and warrant further investigation as clinical safety biomarkers.

Table 6 Results of IDI Analysis comparing each novel biomarker plus AST and CK to AST+CK

Biomarker	Fraction Improved Positive Findings	Fraction Improved – Fraction Diminished Positive Findings	p-value Positive Findings	Fraction Improved Negative Findings	Fraction Improved – Fraction Diminished Negative Findings	p-value Negative Findings	Total IDI	Total IDI p-value
Fabp3	0.725	0.45	3.14E-11	0.775	0.55	<1.00E-17	0.2217	<1.00E-17
Ckm	0.828	0.655	<1.00E-17	0.73	0.46	<1.00E-17	0.2063	<1.00E-17
Myl3	0.688	0.376	2.68E-08	0.818	0.637	<1.00E-17	0.2701	<1.00E-17
sTnI	0.706	0.413	1.0E-09	0.787	0.574	<1.00E-17	0.203	<1.00E-17

Red indicates the improvement is significant at p < 1.00E-04.

4.3. Biomarker correlation with severity of histopathological diagnosis.

As the ROC, IDI, and NRI analyses all examine individual biomarker sensitivity and specificity for SKM degeneration/necrosis in a binary fashion, a Spearman Correlation coefficient was calculated to examine the relationship between the composite histopathology severity score and the individual biomarker response relative to untreated control animals. The composite histopathology score is the highest severity diagnosis (0-4) for skeletal muscle degeneration/necrosis for any muscle recorded for a given animal.

All biomarkers had significant correlation coefficients with histopathology scores (Table 7). A striking pattern emerged as displayed in Figure 3 wherein all biomarkers increased in a score-responsive fashion with increasing severity of SKM injury. However, the novel biomarkers, Ckm, Fabp3, Myl3, and sTnI, had a greater dynamic range (i.e. larger increases) than AST and CK, as well as appeared to better signal minimal injury. For example, Ckm, Myl3 and sTnI increased in a much more dramatic fashion in animals with minimal injury (composite histopathology severity score of 1 for SKM degeneration/necrosis) compared to controls. This apparent sensitivity for recognition of low-grade muscle degeneration is of particular interest to support clinical use of these biomarkers where it is desirable to detect drug-induced SKM injury as soon as possible.

Figure 3 Biomarker change with increasing severity of SKM injury

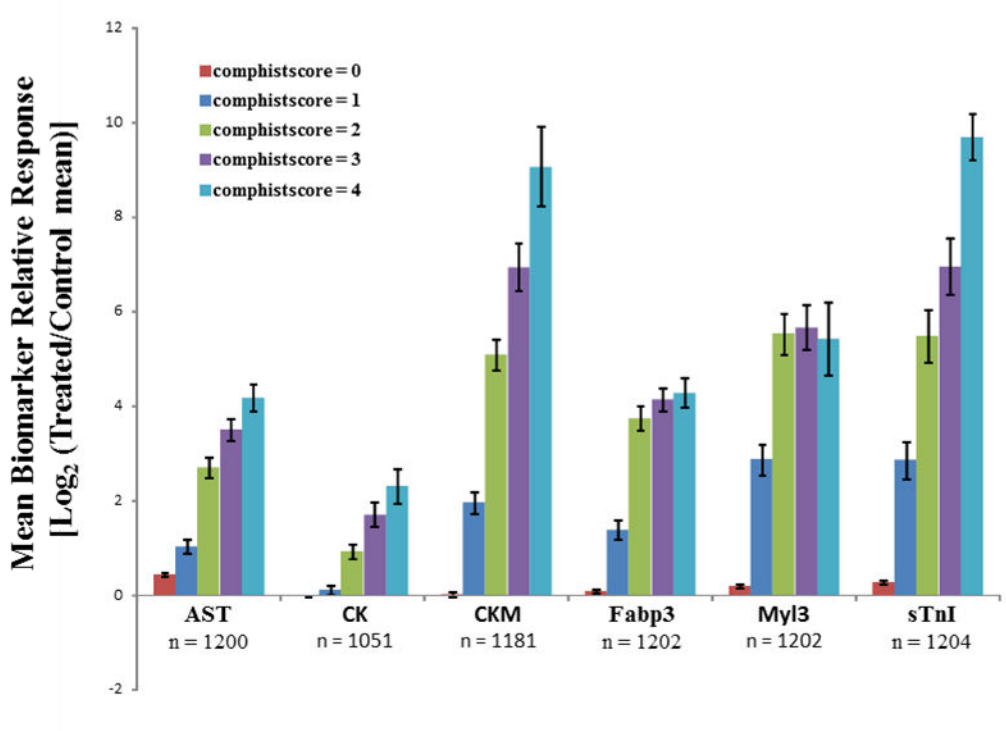


Table 7 Spearman correlation coefficient for biomarker levels and severity of histology score for SKM injury

	Ckm	Fabp3	Myl3	sTnI	AST	CK
Correlation coefficient	0.5372	0.5178	0.4847	0.4567	0.4697	0.3217
p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

4.4. Biomarker specificity for SKM over liver or cardiac tissue

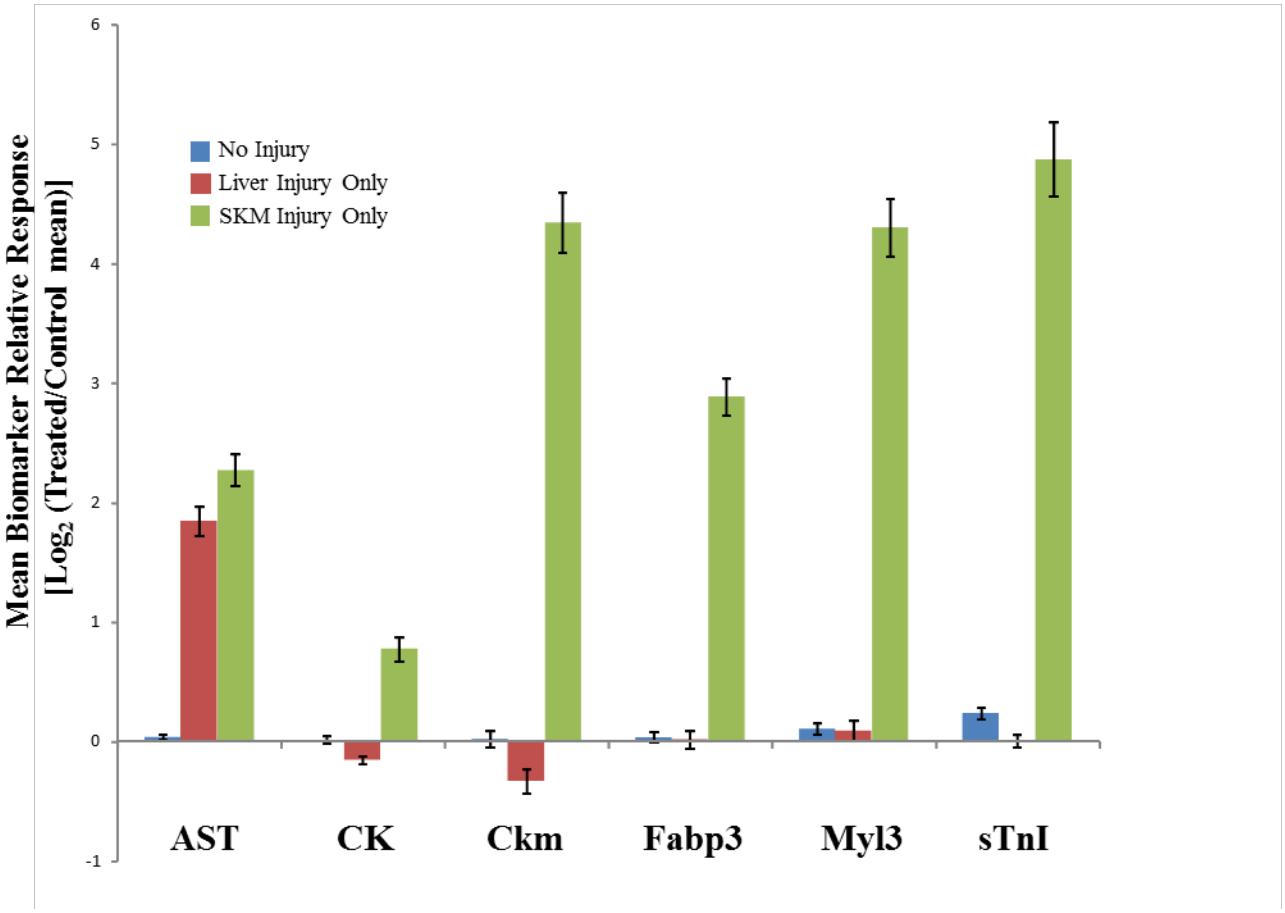
All biomarkers lack specificity in one way or another. For example, the novel biomarkers Myl3, Ckm, and Fabp3 are all expressed in heart tissue, although not in as high concentrations as in skeletal muscle (Table 1). Nonetheless, an increase in these biomarkers could potentially be due to injury to heart, to SKM, or to both tissues concurrently. In addition, AST is highly expressed in the liver, as well as in heart and SKM tissue.

The SKMWG conducted dedicated specificity studies using compounds that cause either liver or cardiac degeneration/necrosis to better understand the SKM biomarker responses in these situations. Unfortunately, most of the compounds selected for cardiac specificity studies also caused microscopically evident SKM degeneration/necrosis, and we were therefore unable to characterize the behavior of the novel biomarkers in situations of cardiac injury without concurrent skeletal muscle injury. The current SKMWG recommendation is therefore to measure cardiac troponin I (cTnI) in addition to the novel SKM biomarkers. Absence of cTnI increases would be interpreted to rule out the cardiac injury as the source of increases in concentrations of novel SKM biomarkers. This is the same strategy currently employed to guide the interpretation of AST and CK activities in situations of striated muscle damage. Conceivably, future studies employing other models to induce cardiac injury (e.g., myocardial infarctions) without affecting skeletal muscle may be useful to clarify the biomarker responses in this scenario.

Histopathology data were recorded to capture microscopic changes in several tissues in addition to SKM. Because some diagnoses recorded for liver and heart were fairly innocuous while others reflected more significant changes, a board-certified pathologist classified each diagnosis as unlikely or likely to be associated with conditions favoring biomarker release (e.g., AST from hepatocytes or AST, Ckm, Myl3, or Fabp3 from cardiomyocytes). Animals thus classified as likely to have liver or cardiac injury favoring biomarker release are shown in the figures below. There were 47 records of “significant” cardiac injury (without concurrent SKM injury) and 25 records of SKM + “significant” cardiac injury. Several liver toxicants were utilized as specificity studies, providing 193 animals with “significant” liver injury (without concurrent SKM injury). Only 3 animals had “significant” liver injury plus SKM injury and were thus omitted from the plot.

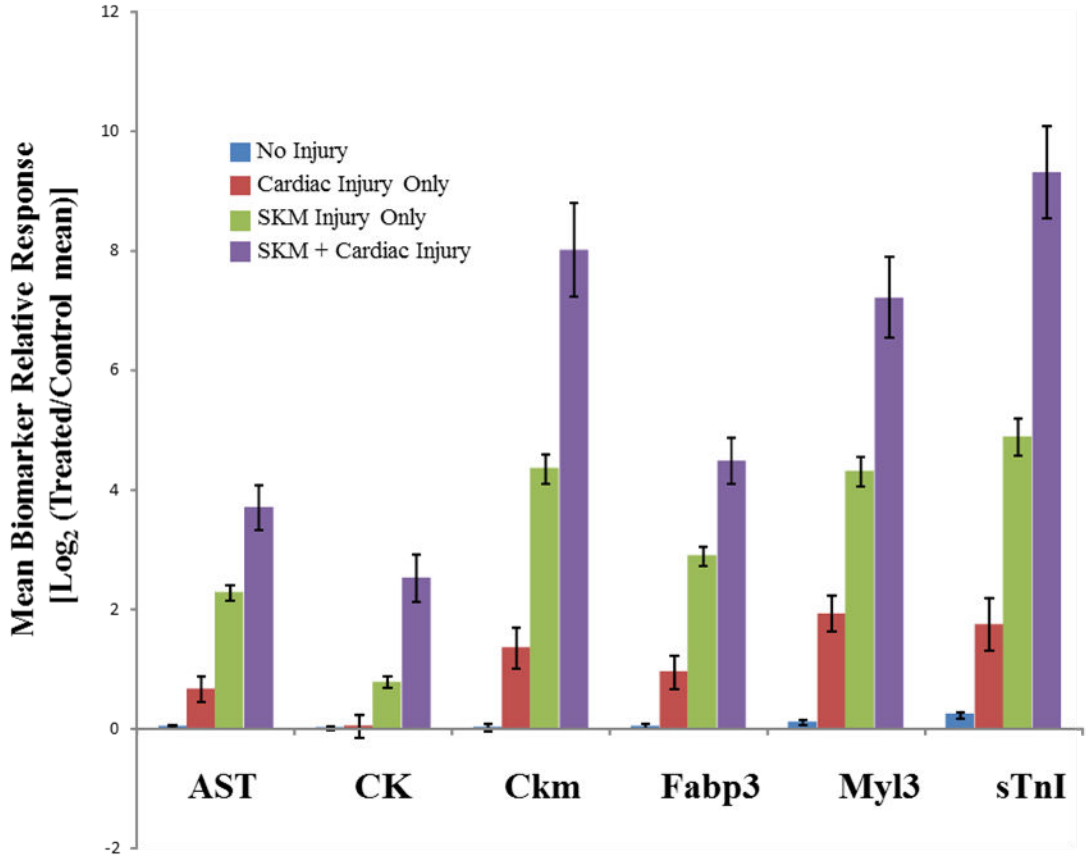
Figure 4 shows plots of biomarker fold-change over control in animals that had either no injury, liver injury only, or SKM injury only. These animals are shown irrespective of treatment (or lack thereof), and are categorized here only on the basis of histopathological diagnosis. In animals with liver injury only, AST values were elevated as expected because AST is released from hepatocytes as well as myocytes. In contrast, the novel SKM biomarkers Ckm, Fabp3, Myl3, and sTnI, show no increase in animals with liver injury only. This further demonstrates the specificity of these biomarkers for SKM injury and indicates one aspect of their improved utility over AST.

Figure 4 Biomarker response in groups with SKM or liver injury



CK, Ckm, Fabp3, and Myl3 are expressed in cardiac myocytes, although in lower amounts than in skeletal myocytes. We again plotted animals classified as having no injury, cardiac injury only, SKM injury only, or cardiac + SKM injury. Results shown in Figure 5 demonstrate anticipated greater-fold increases in animals with SKM injury only (green bars) as compared to cardiac injury only (red bars). However, sTnI, the biomarker with supposed greatest specificity to SKM tissue, showed notable increase in animals with cardiac injury only or SKM+cardiac injury. As noted above, the myocardial toxicants employed in these studies induced varying levels of concurrent skeletal muscle degeneration/necrosis and were therefore not "pure" myocardial toxicants. Therefore, we cannot use this data set to conclude definitively that low-level skeletal myocyte injury was absent in this group of "cardiac injury only" animals. A more definitive statement concerning effects of myocardial damage on the circulating levels of these novel biomarkers must await studies employing more cardiac-specific injury models.

Figure 5 Biomarker values in groups with SKM and/or cardiac injury



4.5. Reversibility studies for monitorability

The objectives of the reversibility studies were to understand how biomarker concentrations changed in animals after discontinuation of treatment and whether these changes in biomarker concentrations were consistent with resolution of SKM degeneration/necrosis as monitored by histopathology in these studies. The rat toxicology studies conducted to address these objectives used a variety of compounds (TMPD, PPAR alpha agonists, Cerivastatin, and proprietary compounds) and were conducted by a number of different companies.

An overview of the biomarker reversibility studies is shown in the Table 8. The sampling times noted in the table refer to the day(s) of the recovery phase where Day 0 of recovery is the next day after cessation of the dosing phase. Studies were designed such that peak injury should be evident at the end of the dosing phase and the start of the recovery phase, and most or all SKM tissue injury should have resolved by the end of the recovery phase.

Results from the reversibility studies are shown in two ways. Figure 6 summarizes mean biomarker levels by corresponding severity of myocyte degeneration/necrosis as observed by histopathology for six studies. Biomarker values and histopathology scores are shown on the first

day of the recovery period and on day 14. Figures 7 and 8 plot biomarker values in individual animals by severity of myocyte degeneration/necrosis as observed by histopathology across time for two representative studies.

Two general conclusions were reached from these experiments evaluating biomarker concentrations in the recovery phase of these rat toxicology studies: (1) biomarker concentrations decreased with time after cessation of dosing and (2) the magnitude of biomarker concentrations at necropsy during the recovery phase is consistent with the severity of SKM degeneration/necrosis detected by histopathology in those animals.

Data for Ckm, Fabp3, Myl3, and sTnI and for the conventional endpoints AST and CK are presented in Figures 7 and 8 for Study 2089 that dosed two SKM toxicants, TMPD and a PPAR-alpha agonist. These are examples of a detailed time course that clearly supports the utility of the novel biomarkers for monitoring recovery from SKM degeneration/necrosis in the rat.

Following a single dose of TMPD (9 mg/kg), myocyte degeneration/necrosis was microscopically evident at high incidence and severity in multiple sampled muscles for the following 6 days. Incidence and severity of microscopic degeneration/necrosis declined by Day 7 and was virtually absent on post-dose Day 14 (minimal degeneration/necrosis still present in one of five sampled muscles in 2 of 5 treated rats). As shown in Figure 7, serum biomarker concentrations increased during the first 5 post-dose days, concurrent with the period of microscopically robust myocyte degeneration/necrosis, then declined by Day 7 and were at baseline levels when assayed on post-dose Day 14.

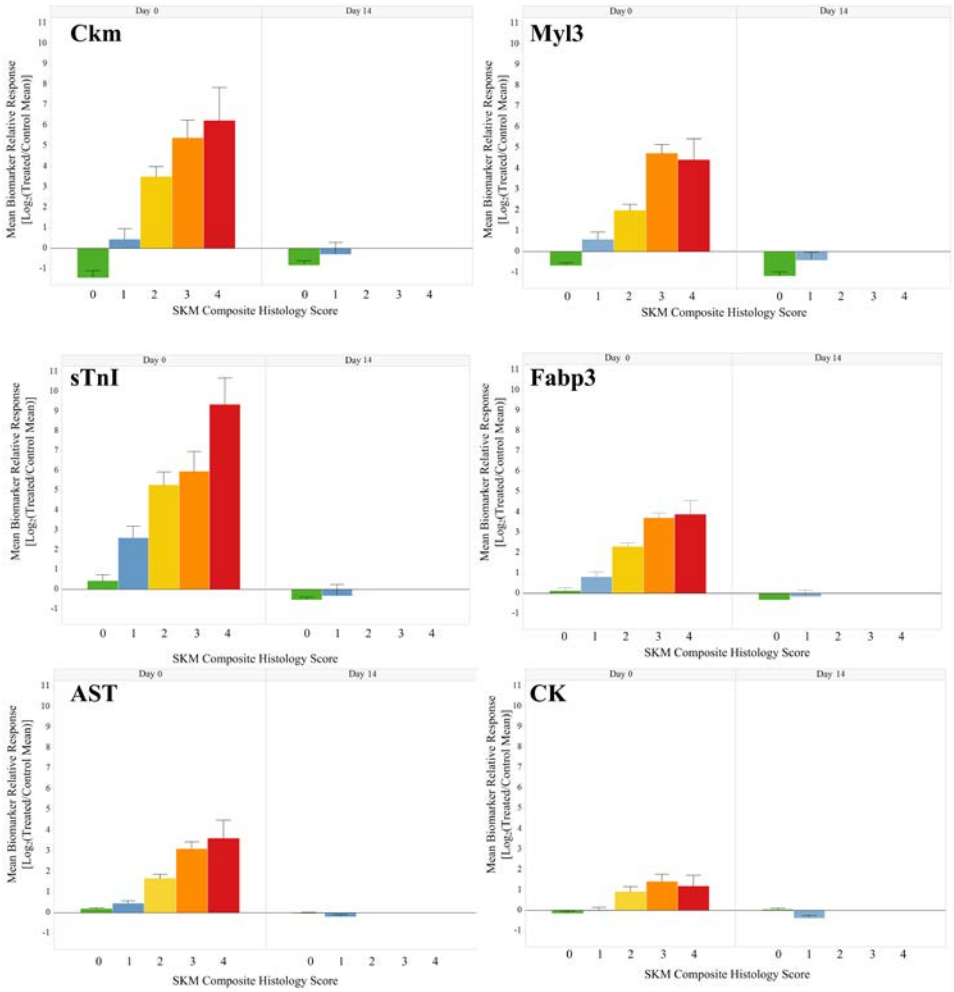
Figure 8 indicates that biomarker values similarly returned to baseline with approximately the same timing as resolution of injury as assessed by histopathology in rats dosed daily for five days with a PPARα agonist. On Days 0-3, biomarker levels were generally elevated, consistent with histopathological findings of active myocyte degeneration/necrosis. On Day 7 and Day 14, both biomarker values and histopathology indicated SKM injury had resolved. CK was an exception to this, and remained elevated on Day 14.

Table 8 Summary of Biomarker Response in Recovery Studies

Compound	Company	Study Number	Sampling time (Days of recovery phase)*
TMPD	Lilly	2089†	0, 1, 2, 3, 5, 7, 14
TMPD	Pfizer	3484	0, 6
PPARα agonist	Lilly	2089†	0, 1, 2, 3, 5, 7, 14
PPARα agonist	Merck	2775†	0, 7, 14
PPARδ agonist	Sanofi-Aventis /Merck	2899†	0, 14
Cerivastatin	Merck	2776†	0, 14
Cerivastatin	Lilly	2089†	0, 1, 2, 3, 5, 7, 14
Pfizer Compound	Pfizer	2200†	0, 15
BMS Compound	BMS	1143	0, 6

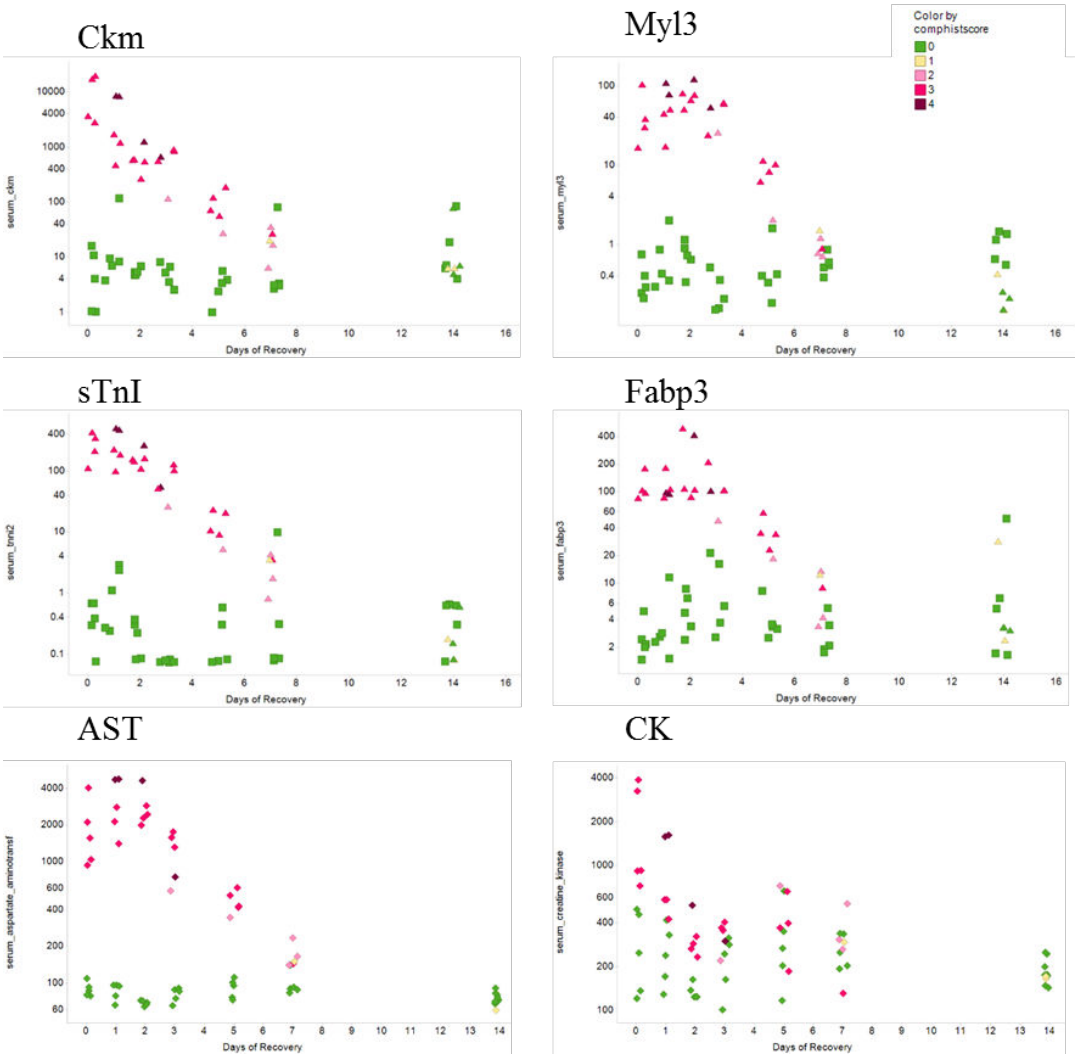
*Day 0 of recovery is the first day following cessation of the dosing phase of the study.
†: study included in Figure 6.

Figure 6 **Plot of mean biomarker results from seven recovery studies at the first day of recovery and last day of recovery**



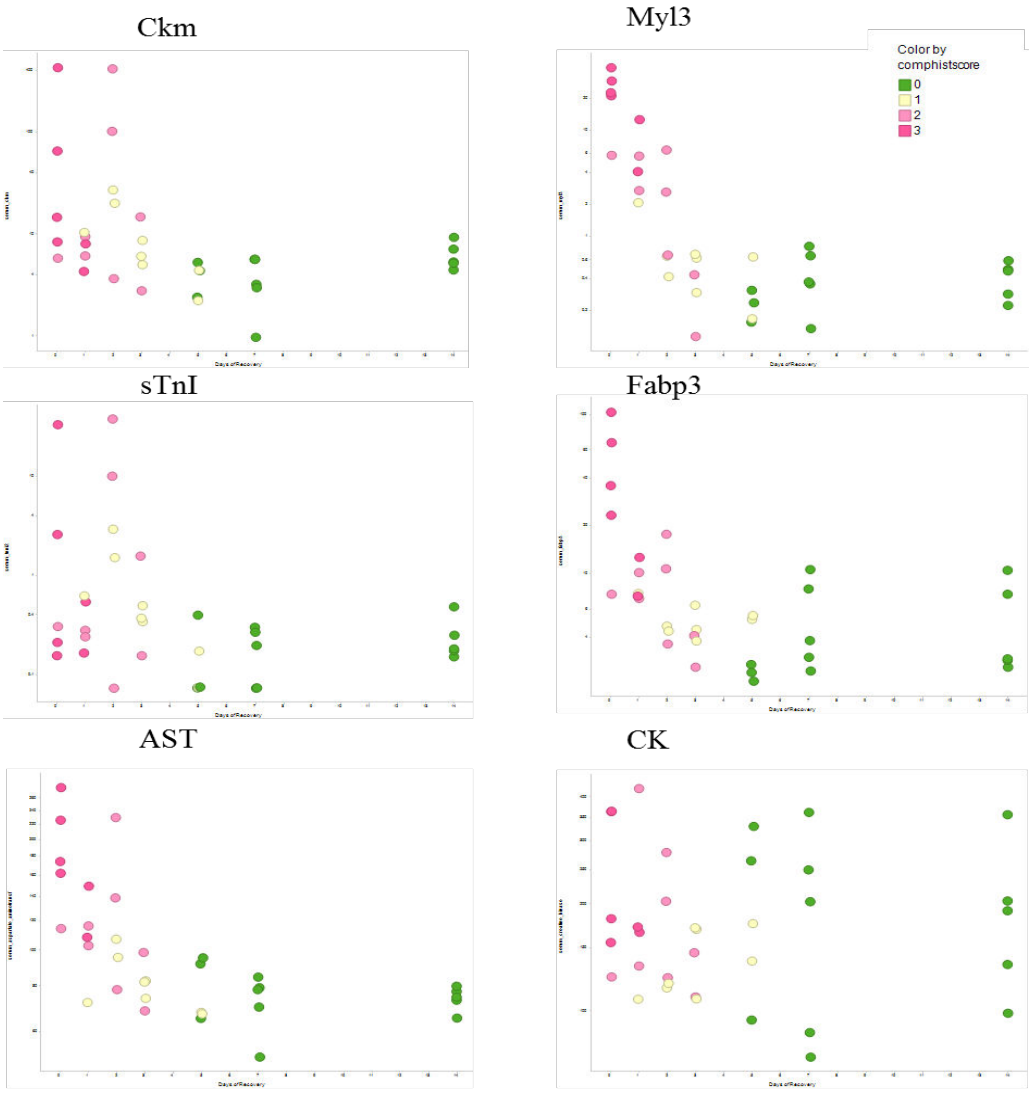
In each panel of Figure 6, the mean biomarker relative response for all SKM degeneration/necrosis histology scores is plotted for Day 0 and 14 after cessation of drug administration. The y-axis is the mean biomarker relative response and the error bars represent the standard error of the mean. The x-axis is the SKM degeneration/necrosis composite histology score [0 (within normal limits; depicted using a blue bar) to 4 (severe SKM degeneration/necrosis; depicted using a red bar)]. The relative response was calculated by normalizing each animal to the concurrent timepoint vehicle control group mean and then taking the Log_2 of this ratio and plotting the mean for each histology score of all treated animals in these studies.

Figure 7 Plot of serum results for TMPD recovery study (Study 2089)



In each panel of Figure 7, the severity scores for skeletal myocyte degeneration/necrosis are presented as a color range from green being no detectable SKM degeneration/necrosis to dark red being severe SKM degeneration/necrosis. The symbol shape indicates dose group: squares are control animals and triangles are animals administered TMPD. The X-axis presents days following end of dosing phase, for which 0 represents 24 hours after the one and only administration of TMPD (9 mg/kg) to the rats. Symbols are "jittered" along the X-axis so that symbols do not lie directly on top of one another. The Y-axis is a log scale and presents absolute biomarker concentration for individual animals.

Figure 8 Plot of serum results for PPAR-alpha agonist recovery study (Study 2089)



In each panel of Figure 8, the severity scores for skeletal myocyte degeneration/necrosis are presented as a color range from green being no detectable SKM degeneration/necrosis to dark red being severe SKM degeneration/necrosis. Rats were dosed daily for five days with either 300 mg/kg PPAR-alpha agonist or vehicle. All animals regardless of dose group (control or treated) are shown as circles. The X-axis presents days following end of dosing phase, for which 0 represents 24 hours after the final administration of drug to the rats. Symbols are "jittered" along the X-axis so that symbols do not lie directly on top of one another. The Y-axis is a log scale and presents absolute biomarker concentration for individual animals.

4.6. Skeletal muscle biomarkers in aged rats

A study was designed to assess the sensitivity and specificity of Ckm, Fabp3, Myl3, and sTnI in young (8-10 week) and aged (1 year old) male Sprague-Dawley rats after drug-induced skeletal muscle or liver injury. This information is especially valuable in support of the future use of these biomarkers in aged clinical populations.

TMPD was used at 1 and 5 mg/kg to specifically induce muscle injury, and acetaminophen (APAP) was used at 1400 mg/kg in separate cohorts of rats to induce liver injury. Histopathology confirmed the presence of skeletal muscle injury in the TMPD treated rats and liver injury in the APAP treated subjects. Analysis of the conventional clinical chemistry panel revealed no significant differences in the basal activities of CK, ALT, or AST enzyme activities in the young versus aged vehicle control groups. AST and ALT, but not CK, were elevated in both the 5 mg/kg TMPD and the APAP treated young and aged groups. The mean AST and ALT levels in the TMPD and APAP treated groups were approximately two fold greater in the aged versus the young rats. The average serum concentrations of the proposed skeletal muscle injury biomarkers sTnI, Fabp3, Myl3, and Ckm were numerically higher in the aged rat vehicle control group compared to the young vehicle control group but this difference was not statistically significant. Importantly, both the young and aged treated groups showed significant increases in the serum concentrations of sTnI, Fabp3, Myl3 and Ckm compared to their respective vehicle control groups when treated with the myotoxicant TMPD, but not when treated with the hepatotoxicant APAP. Furthermore, the novel muscle biomarker serum concentrations measured in the TMPD-treated aged rats were significantly greater than those in the TMPD-treated young rats.

The overall conclusion from this study is that while the baseline serum concentrations of the SKM biomarkers were numerically higher in aged than in young rats, these biomarkers still increased significantly in response to TMPD-induced skeletal muscle injury and did not respond to APAP-induced liver injury. In marked contrast, the conventional serum biomarkers AST and ALT were increased in response to both TMPD-induced muscle injury and to APAP-induced liver injury. Serum CK activity did not increase in response to either treatment. This indicates that the novel SKM biomarkers are not confounded by age and may be more specific for drug-induced skeletal muscle injury than AST and CK.

4.7. Assessing skeletal muscle injury with the novel biomarkers in canines

To begin to address translation of these biomarkers to species other than rat, a skeletal muscle toxicity study was conducted in dogs to assess the ability of the exploratory skeletal muscle injury biomarkers to monitor drug-induced skeletal muscle injury and leverage the biomarker data to demonstrate recovery of the skeletal muscle injury. Cross reactivity of the biomarker assays was first demonstrated using dog tissue lysates.

A development compound (Merck X) was previously shown to induce skeletal muscle injury in dogs in a short term study. A chronic study was designed using data obtained from interim skeletal muscle biopsies as well as exploratory skeletal muscle injury biomarkers to monitor the onset of the skeletal muscle injury. Dogs were treated with Merck X orally for twelve weeks, followed by a twelve-week drug free recovery period. Interim plasma samples were collected prior to muscle biopsy to ensure that elevations were due to the drug-induced skeletal muscle injury and not due to the biopsy procedure itself. Treated animals were divided into four subgroups according to their final necropsy day and biopsy schedules. Subgroup A (controls and Merck X at 2 mg/kg/day) animals were sacrificed at study day 29; Subgroup B and D (controls, Merck X at 2 mg/kg/day and 8 mg/kg/day) animals were sacrificed at study day 85 at the end of

the dosing period, Subgroup C (controls, Merck X at 8 mg/kg/day) animals were sacrificed at study day 168 after 12 weeks of dosing and a 12 week recovery period.

sTnI, Myl3, and Ckm showed treatment-related increases in Subgroup C and Subgroup D with Merck X treatment at the 8 mg/kg/day dose level starting as early as day 15, reaching a maximum around week 8 and decreasing to control levels in recovery animals at week 24. Increases in the exploratory skeletal muscle biomarkers correlated with drug-induced skeletal muscle degeneration observed in the interim biopsy samples as well as the final necropsy histopathology samples, and in some animals, were more sensitive in detecting the skeletal muscle injury than AST and/or CK. These findings demonstrated the utility of these skeletal muscle injury biomarkers to monitor the onset of test article-related skeletal muscle injury in a chronic safety study in non-rodents, as well as to monitor the recovery of the induced muscle degenerative lesions following cessation of treatment.

5. Conclusion

The aim of this work is to demonstrate the biological relevance and toxicological responsiveness of the SKM injury biomarkers sTnI, Myl3, Ckm, and Fabp3 in nonclinical species to support future qualification for clinical use. The nonclinical evidence presented here supports our current proposal that sTnI, Myl3, Ckm, and/or Fabp3 are suitable for voluntary use in combination with CK and AST, in regulatory safety studies in rats to demonstrate that drug-induced skeletal muscle degeneration/necrosis is monitorable. These data, with additional data to be generated by the drug development sponsor in a second toxicology species as well as in humans, could be used to deploy exploratory biomarker monitoring strategies in early clinical trials to ensure patient safety and enable drug development to proceed.

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