Biomarkers, June 2008; 13(4): 364–376

informa
healthcare

Correlation analysis of gene expression and clinical chemistry to identify biomarkers of skeletal myopathy in mice treated with PPAR agonist GW610742X

W. M. CASEY[1], T. BRODIE[2], L. YOON[1], H. NI[1], H. L. JORDAN[1], & N. F. CARIELLO[1]

[1]*GlaxoSmithKline, US Research & Development, Safety Assessment, Five Moore Drive Research Triangle Park, NC 27709, USA and* [2]*GlaxoSmithKline, UK Research & Development, Safety Assessment, The Frythe, Welwyn AL6 9AR, UK*

Abstract
Data from individual animals were used to identify genes in mouse skeletal muscle whose expression correlated with a known serum marker of skeletal myopathy, creatine kinase activity (CK), after treatment with a peroxisome proliferator-activated receptors (PPAR) agonist, GW610742X. Six genes had correlation coefficients of ≥ 0.90: *Mt1a* (metallothionein 1a), *Rrad* (Ras-related associated with diabetes), *Ankrd1* (ankyrin repeat domain 1), *Stat3* (signal transducer and activator of transcription 3), *Socs3* (suppressor of cytokine signalling 3) and *Mid1ip1* (Mid1 interacting protein 1). The physiological function of these genes provides potentially useful information relating to the mechanism of PPAR-induced skeletal myopathy, with oxidative stress and disruption of glycolysis most closely associated with myopathic damage. Some of the muscle genes most highly correlated with serum CK in mice also appear to be good indicators of PPAR-induced myopathy in rat skeletal muscle, demonstrating the translational potential of this approach. This study clearly shows the utility of using correlation analysis as a simple tool for identifying novel biomarkers and investigating mechanisms of toxicity.

Keywords: *PPAR, oxidative stress, glycolysis, gene expression, myopathy, biomarker, correlation*

(Received 18 July 2007; accepted 8 January 2008)

Introduction

Peroxisome proliferator-activated receptors (PPAR) are a family of ligand-activated nuclear hormone receptors which regulate the transcription of genes involved in energy homeostasis (reviewed in Willson et al. 2000, Desvergne et al. 2006, Feige et al. 2006, Guri et al. 2006). Three types of PPAR receptors have been identified in mammals: α, β/δ (referred to as PPARδ from here on) and γ. PPARα and δ regulate the trafficking and metabolism of fatty acids, while PPARγ functions as a transcriptional regulator of both adipogenic and lipogenic programmes and has also been implicated in whole-body glucose homeostasis and insulin sensitivity.

Correspondence: W. M. Casey, Glaxo SmithKline, Five Moore Dr. 9.2023, RTP NC 27709, USA. Tel: 919 483 3612. Fax: 919 483 6858.

ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.1080/13547500801903545

PPARα was initially identified as a mediator of the peroxisome proliferative response in rodent hepatocytes after exposure to a non-genotoxic carcinogen (Issemann & Green 1990). PPARδ and PPARγ were subsequently identified by sequence homology, but do not mediate hepatic peroxisome proliferation. Work using PPARα-null mice has demonstrated that hepatic proliferative response and associated hepatocarcinogenesis requires the PPARα receptor (Chen et al. 2000, Shankar et al. 2003, Anderson et al. 2004). The expression of PPARα is high in tissues with an elevated capacity for fatty acid oxidation, such as liver, heart, skeletal muscle, brown fat and kidneys, while PPARδ is more ubiquitously expressed. Activation of the PPARα receptor with synthetic ligands (i.e. fibrates) has been used as a pharmacological approach in the treatment of hypolipidaemia, although clinical use of fibrates has been linked to skeletal myopathy (Hodel 2002, Peraza 2006).

Safety biomarkers in both clinical and preclinical studies can be valuable tools for pharmaceutical development and disease management. The ability to identify novel biomarkers offers the potential to enhance risk assessment and reveal biochemical mechanisms of action. A common problem encountered in preclinical biomarker development is the large interindividual variability, even though the animals (rodents) are putatively isogenic and environmental conditions are tightly controlled. Inter-animal variability, coupled with the relatively small number of animals in a group, often makes it difficult to achieve statistical significance among treatment groups when toxicological events are rare or weakly expressed.

Correlation analysis is a multivariate technique which uses the entirety of the data to assess the relationship between two or more variables. Since the correlation coefficient is based on the slope of a regression line, a single extreme measurement is capable of significantly changing the value of the correlation. Consequently, fewer animals are needed to identify potential biomarkers via correlation as compared with standard group-wise methods (i.e. a single affected animal in the treated group is enough to identify a correlative biomarker, whereas a standard *t*-test with the same dataset would yield a poor *p*-value for any marker associate with only one affected animal).

In this study we identified gene expression patterns from the quadriceps muscle of individual male mice treated with myopathic doses of the PPARα/δ dual agonist GW610742X which correlated with an established biomarker of muscle damage, serum creatine kinase activity (CK). A wide range of myopathic response was elicited by using two doses of GW610742X in both wild-type and PPARα knockout mice, as recent work in our lab has shown that the myopathic response elicited by GW610742X is more pronounced in wild-type compared with PPARα knockout mice (data presented herein).

Genes identified using this method could serve as biomarkers of PPAR-induced skeletal myopathy. In addition, the physiological function of proteins encoded by genes could provide insight into the underlying myopathic mechanism(s). The translational capability of this approach was examined by examining the expression of punitive myopathy marker genes, identified in quadriceps muscle of male mice, and in the soleus muscle of female rats treated with a myopathic dose of GW610742X over a 7-day time course. Although one outcome of this study was to discover marker genes associated with PPAR-mediated myopathy, the primary objective was to demonstrate the general utility of correlation analysis for the identification of biomarkers. This methodology, which is applicable to any analytical data, could greatly reduce both the number of animals and time required to develop biomarkers of safety or efficacy.

Materials and methods

Test compound

GW610742X is a high-affinity agonist for the PPARδ receptor (Sznaidman et al. 2003). Selectivity and potency ligand-binding studies demonstrated EC_{50} values for GW610742X of 0.03 μM for murine PPARΔ, versus 8.8 μM for PPARα and >10 μM for PPARγ (the highest concentration tested against this receptor). The plasma C_{max} of GW610742X in both mice and rats given oral doses of 100 mg kg^{-1} significantly exceeds the EC_{50} of PPARα, often by a factor of 25 (unpublished data). Accordingly, significant PPARα agonism, in addition to PPARδ agonism, is expected in the current study. The vehicle for GW610742X was 0.5% hydroxypropyl methylcellulose in reverse osmosis-treated water.

Treatment

Mouse study. Groups of five male PPARα knockout mice (B6.129S2-*Ppara*tm1N12) and their corresponding wild-type (WT) control (C57BL/6) mice were given 0 (vehicle), 100 or 250 mg kg^{-1} GW610742X once daily for 10 days by oral gavage. Animals were euthanized on day 10 and skeletal muscles were flash frozen in liquid nitrogen and stored at −80°C. GW610742X-induced myopathy is dose-dependent and appears to be mediated primarily through the PPARα receptor (GW610742X is expected to bind both the PPARα and δ receptors at doses used in this study; see Test compound above). As stated above, this study was designed to elicit a wide range of myopathic responses.

Rat study. Two groups of female Sprague Dawley rats ($n = 5$) were treated daily by oral gavage with 100 mg kg^{-1} daily GW610742X for 2, 4 or 6 days. Animals were euthanized on the morning following the last treatment (days 3, 5 and 7) and tissues were collected and processed as described above.

Serum chemistry

At study termination, blood samples were collected from the abdominal vena cava while the animals were under isoflurane anaesthesia. Creatine kinase activity was measured on the lympus AU640e analyzer according to the method described by Szasz (1976).

Histopathology

Skeletal muscles were fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned at 5 μm and stained with haematoxylin and eosin. Muscle sections were examined by light microscopy by a pathologist and observations of myopathic lesions were recorded.

RNA analysis

Frozen muscle samples were lysed in Trizol reagent (Gibco BRL, Life Technologies) and RNA was precipitated with isopropanol and the pellet was washed with 80% ethanol and resuspended in diethyl pyrocarbonate-H_2O. RNA quality was checked by an Agilent Bioanalyzer**.** RNA was purified through a Qiagen column and quantified

using OD_{260} absorbance. RNA samples were analyzed using Affymetrix GeneChip® (MOE430A or RAE230A) with standard protocols.

Affymetrix GeneChip analysis

PowerArray software (http://www.niss.org/PowerArray/) was used to extract data from the GeneChip® image files. Data were examined using BioConductor and Principle Component Analysis for quality control.

Correlation between CK and gene expression data

Previous studies on PPAR-induced myopathy in rodents have indicated that CK elevations are more closely associated with pathological observations of skeletal myopathy in mice, whereas aspartate aminotransferase appears to be a more suitable marker in rats (unpublished data). Temporal considerations are also important in our study design as the onset of myopathy first occurs in one of the larger muscle groups (quadriceps), which facilitates the association of elevated CK levels with this muscle (unpublished data). Serum CK levels at later time points would start to reflect the cumulative damage to numerous muscles, and would therefore not correlate well with any specific muscle group. Consequently, we hypothesized that a significant portion of the serum CK activity in the present study would originate from quadriceps muscle given its large mass and high degree of myopathic damage. Correlation analysis of serum CK and quadriceps gene expression data was performed in Microsoft Excel using the 'CORREL' function (Pearson's r), comparing CK values for each animal with $\log_2$- transformed signal intensities from the $\sim$18 000 Affymetrix probe sets for each animal. A correlation coefficient (r) of ≥ 0.90 was used as the cut-off for this analysis to ensure a robust selection of probe sets.

Results and discussion

Affymetrix GeneChip® quality control analysis

Mouse study. A total of 30 MOE430A GeneChip® ($n = 5$ per group, six groups) were processed. After quality control (QC) analysis, results from one animal number (WT control) failed to meet QC criteria and was therefore excluded, leaving 29 animals for further analysis.

Rat study. A total of 30 RAE230A GeneChip® ($n = 5$ per group, six groups) were processed. After QC analysis, results from two animals (GW610742X treated day 3 and control day 5) failed to meet QC criteria and were therefore excluded, leaving 28 animals for further analysis.

Clinical chemistry and myopathy scores from individual animals

Myopathy was characterized by varying degrees of myofibril degeneration, accompanied by intrafibrillar and interstitial inflammatory cell infiltrates interstitial oedema and occasional myofibre regeneration. The most severely affected muscle was the quadriceps, followed by the intercostals, gastrocnemius, soleus and diaphragm in descending order of severity. A low incidence of myofibre regeneration was noted in

some groups, but it did not bear a clear relationship to treatment. Based on histopathological evaluation, numeric values (myopathy scores) for severity grades of skeletal myopathy were assigned as follows: minimal = 1, mild = 2, moderate = 3, marked = 4. Myopathy scores for each muscle group in each animal along with individual animal serum CK data are presented in Table I. Myopathy was noted in only one high-dose PPAR knockout animal, whereas one wild-type animal in the 100 mg kg^{-1} daily group and all five animals in the high-dose group had myopathic damage. This represents a significant difference between the genotypes and indicates that the PPARα receptor plays a major role in GW610GW610742X-induced myopathy. This heterogeneous response in mice with different genetic backgrounds proved to be ideal for the current study in that it provided the type of

Table I. Individual animal clinical chemistry and myopathy data. Clinical chemistry values for individual animals are reported in U L^{-1}. Myopathy scores are based on severity grade: minimal = 1, mild = 2, moderate = 3, marked = 4.

			Myopathy score				
GT dose (mg)	Animal no.	CK (U L^{-1})	IC	Sol	Quad	Gast	Dia
Knockout							
0	7	68	0	0	0	0	0
0	10	133	0	0	0	0	0
0	19	81	0	0	0	0	0
0	21	121	0	0	0	0	0
0	27	93	0	0	0	0	0
100	11	259	0	0	0	0	0
100	16	91	0	0	0	0	0
100	1	78	0	0	0	0	0
100	2	120	0	0	0	0	0
100	3	302	0	0	0	0	0
250	5	267	0	0	0	0	0
250	12	177	0	0	0	0	0
250	13	1942	0	0	0	0	0
250	4	288	0	0	0	0	0
250	9	1922	0	0	2	0	0
Wild type							
0	48	101	0	0	0	0	0
0	54	97	0	0	0	0	0
0	66	160	0	0	0	0	0
0	67	108	0	0	0	0	0
100	49	150	0	1	0	0	0
100	53	1381	0	0	2	0	0
100	57	149	0	1	0	0	0
100	58	393	0	0	0	0	0
100	59	279	0	0	1	0	0
250	46	2012	2	1	4	0	0
250	47	1612	2	0	3	2	0
250	62	3030	3	1	4	2	2
250	64	1881	2	0	3	1	0
250	72	827	2	0	3	0	0

GT, genotype; CK, creatine kinase; IC, intercostals; Sol, soleus; Quad, quadriceps; Gast, gastrocnemius; Dia, diaphragm.

non-homogeneous response often seen in clinical studies, and which highlights the utility of using correlation analysis for this type of response.

There is a close association between elevated CK activity and the presence of skeletal myopathy in quadriceps muscle, although it is difficult to calculate meaningful correlation coefficients due to the disparate nature of the analyses (CK is quantitative while myopathy scores are semiquantitative). CK was elevated in all but one animal (no. 59, WT 100 mg kg^{-1} daily GW610742X) with myopathy noted in the quadriceps muscle (Figure 1). Animal no. 13 (PPARα knockout (KO), 250 mg kg^{-1} daily GW610742X) showed increased CK levels, but myopathy was not noted in any muscle group examined.

Most highly correlated genes

Correlation analysis of Affymetrix GeneChip® data from quadriceps muscle and serum CK values revealed six genes which had correlation coefficients of ≥ 0.90: *Mt1a* (metallothionein 1a), *Rrad* (Ras-related associated with diabetes), *Ankrd1* (ankyrin repeat domain 1/cytokine inducible nuclear factor), *Stat3* (signal transducer and activator of transcription 3), *Socs3* (suppressor of cytokine signalling 3) and *Mid1ip1* (Mid1 interacting protein 1), as given in Table II.

Metallothionein (MT) is a highly conserved, low molecular weight, cysteine-rich protein which shares important features with glutathione (GSH). The sulfhydryl

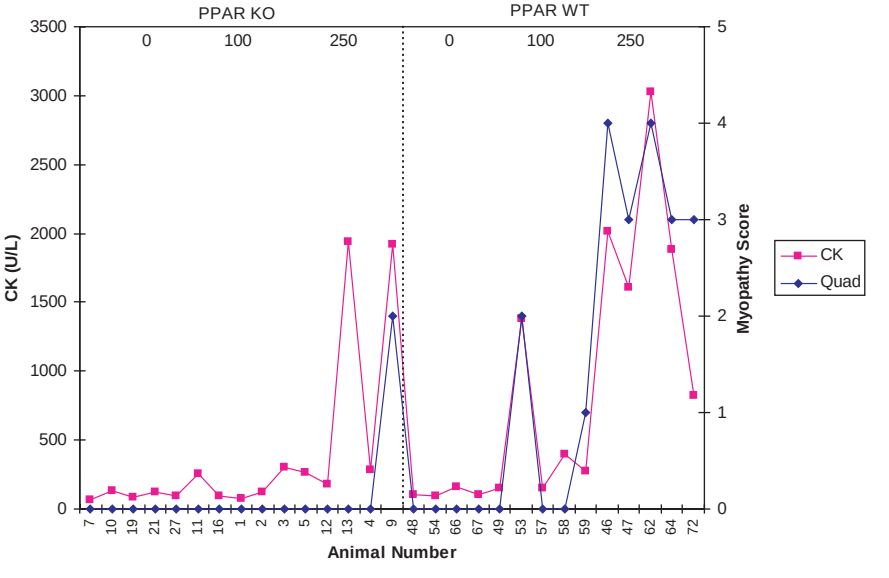

Figure 1. Serum chemistry vs quadriceps myopathy score from individual animals. Selected serum chemistry values are plotted against quadriceps (Quad) myopathy score for each animal used in this report. Serum chemistry values are reported as U L^{-1}. Numeric values (myopathy scores) for severity grades of skeletal myopathy were assigned as follows: minimal = 1, mild = 2, moderate = 3, marked = 4. Animal numbers are arranged by genotype and dose from left to right, starting with PPAR knockout (KO) groups ($n = 5$, control, 100 mg kg^{-1} daily, 250 mg kg^{-1} daily GW610742X) followed by PPAR wild-type (WT) groups (control, 100 mg kg^{-1} daily, 250 mg kg^{-1} daily GW610742X). One WT control animal was excluded from gene expression analysis as a technical outlier and was therefore excluded from the remainder of the analysis, resulting in an $n = 4$ for the WT control group. CK, creatine kinase.

Table II. Correlation of serum creatine kinase (CK) and quadriceps Affy data from GSK Study M41350. Correlation analysis of CK and gene expression data was performed in Microsoft Excel using the 'CORREL' function, comparing CK values for each animal with signal intensities (Omics Studio, $\log_2$ transformed) from each of the ~18 000 Affymetrix probe sets for each animal. Probe sets were ranked based on their correlation coefficients (r). Genes with r-values ≥ 0.90 are listed in the Table below.

Gene symbol	Gene title	Affy ID	r
Mt1	metallothionein 1	1422557_s_at	0.94
Rrad	Ras-related associated with diabetes	1422562_at	0.94
Ankrd 1	ankyrin repeat domain 1	1420992_at	0.92
Stat3	signal transducer and activator of transcription 3	1460700_at	0.90
Socs3	suppressor of cytokine signaling 3	1416576_at	0.90
Mid1ip1	Mid1 interacting protein 1	1416840_at	0.90

groups of GSH and MT serve as the major intracellular thiolate pool. Thiolate groups in MT are preferential targets for H_2O_2 compared with GSH (Quesada et al. 1996), and kinetic studies demonstrated that MT was 38.5-fold more potent than GSH on a molar basis in preventing HO-induced DNA degradation (Abel & Ruiter 1989). Zinc, which is released from MT under conditions of oxidative stress, has antioxidant properties by inhibiting HO· formation through antagonism of redox-transition metals (Maret 1994, Jiang et al. 1998, Powell 2000).

Ras-associated with diabetes (RRAD) is a member of the Ras-related RGK (RAD, GEM and KIR) family of small GTP-binding proteins. This cytosolic protein is most highly expressed in the heart, lung and skeletal muscle (Reynet & Kahn 1993). Rrad was originally identified as being overexpressed in skeletal muscle of patients with type 2 diabetes mellitus (Reynet & Kahn 1993, Moller et al. 1996), and its expression is positively regulated by insulin (Laville et al. 1996). Adipocytes and muscle cells in culture in which Rrad is overexpressed exhibit a reduction in the rate of insulin-stimulated glucose uptake (Moyers et al. 1996). Recently, a potential synergistic interaction has been demonstrated between increased expression of Rrad and high-fat diet in development of insulin resistance and altered lipid metabolism in type 2 diabetes (Ilany et al. 2006). Taken together, these reports indicate that RRAD appears to be an important negative regulator of glucose utilization in the muscle.

Ankyrin repeat domain 1 (ANKRD1) is also known as 'cytokine inducible nuclear factor' and 'cardiac adriamycin-responsive protein (CARP)'. Ankrd1 expression is increased in the mouse model of muscular dystrophy with myositis (Witt et al. 2004). This cardiac-restricted nuclear protein is constitutively expressed and its mRNA level is extremely sensitive to doxorubicin (Jeyaseelan et al. 1997). Moreover, rapid elimination of ANKDR1 by adriamycin exposure has been hypothesized to contribute to the cardiac-specific toxicity of adriamycin and the development of cardiomyopathy (Jeyaseelan et al. 1997).

Signal transducer and activator of transcription, family member 3 (STAT3) is a member of a class of transcription factors bearing SH2 domains that become activated upon tyrosine phosphorylation. STATs are often activated by members of the JAK family of protein-tyrosine kinases in response to cytokine stimulation, and *Stat3* transcription can be induced by H_2O_2 and superoxide in numerous tissues (Arany et al. 2006, Li et al. 2006, Fernandez et al. 2007).

The gene, suppressor of cytokine signalling, family member 3 (*Socs3*), encodes a member of the STAT-induced STAT inhibitor (SSI), also known as suppressor of

cytokine signalling (SOCS). These SOCS family members are cytokine-inducible negative regulators of cytokine signalling. SOCS proteins suppress the cellular responses to inflammatory cytokines including interleukin (IL)-6, IL-10 and interferon-γ. Transcription of *Socs3* has also been show to be induced by STAT3 (Qing & Stark 2004). SOCS molecules, SOCS-1 and SOCS-3, interfere with the cell's ability to respond to leptin, which increases leptin resistance (Bjorbaek, et al. 1998, 1999). SOCS-3 also blocks the response to insulin, thereby increasing insulin resistance (Emanuelli et al. 2001, Senn et al. 2003, Farrell 2005).

Mid1 interacting protein 1 (*Mid1ip*) is a poorly characterized gene which is induced by dexamethasone (Kolbus et al. 2003), a response that may be related to oxidative stress associated with this drug (Schafer et al. 2005).

Genes in the quadriceps which are highly correlated with serum CK levels encode proteins that share overlapping physiological functions relating to oxidative stress (*Mt1a, Stat3, Mid1ip*), cytokine response (*Ankrd1, Stat3, Socs3*) and decreased utilization of glucose (*Rrad, Socs3*). Induction of these genes coincident with serum CK elevations implicates oxidative stress in conjunction with decreased glycolytic activity as a possible mechanism of PPAR-induced myopathy. Graphs of CK values and gene signal intensities for the two genes most highly correlated with serum CK (*Mt1a* and *Rrad*) are provided in Figure 2, illustrating the relative changes in signal intensities from these analytes.

Gene expression versus myopathy in mouse quadriceps muscle.

Genes showing a strong correlation with serum CK ($r \geq 0.90$) also demonstrated a good relationship with myopathy. Increased mRNA levels were noted for all six genes in each animal where myopathy of the quadriceps muscle was noted, with the exception of animal no. 59 (WT 100 mg kg^{-1} daily GW610742X) (Figure 3). Animal no. 59 was also the exception to the association between serum CK and myopathy. The myopathy noted in this animal was graded as minimal (myopathy score 1) and other serum markers associated with muscle damage were unchanged (aldolase and aspartate aminotransferase, data not shown), perhaps indicating that the myopathic damage observed in animal no. 59 may be a 'background' lesion and not related to drug treatment.

A clear association can be seen when plotting myopathy scores and Mt1a expression levels from quadriceps of individual mice (Figure 4). With the exception of animal no. 59, every incidence of myopathy was matched by increased expression of Mt1a in the quadriceps muscle. In one case, animal no. 13 (PPARα KO, 250 mg kg^{-1} daily GW610742X), there was increased expression of Mt1a but no myopathy was noted by microscopic examination, although serum CK was also elevated in this animal (see Figure 1). This discrepancy may result from the limited tissue area examined microscopically, as myopathic lesions are not always uniformly distributed (i.e. myopathic lesions were present but not in the areas examined).

Myopathy marker gene expression in GW610742X-treated rats

In order to determine if these findings could translate to another rodent model and muscle group, we examined myopathy and gene expression in the soleus of Sprague Dawley rats treated with a myopathic dose of GW610742X over a 7-day time course. The soleus is a small muscle, and one of many in rats similarly affected by treatment

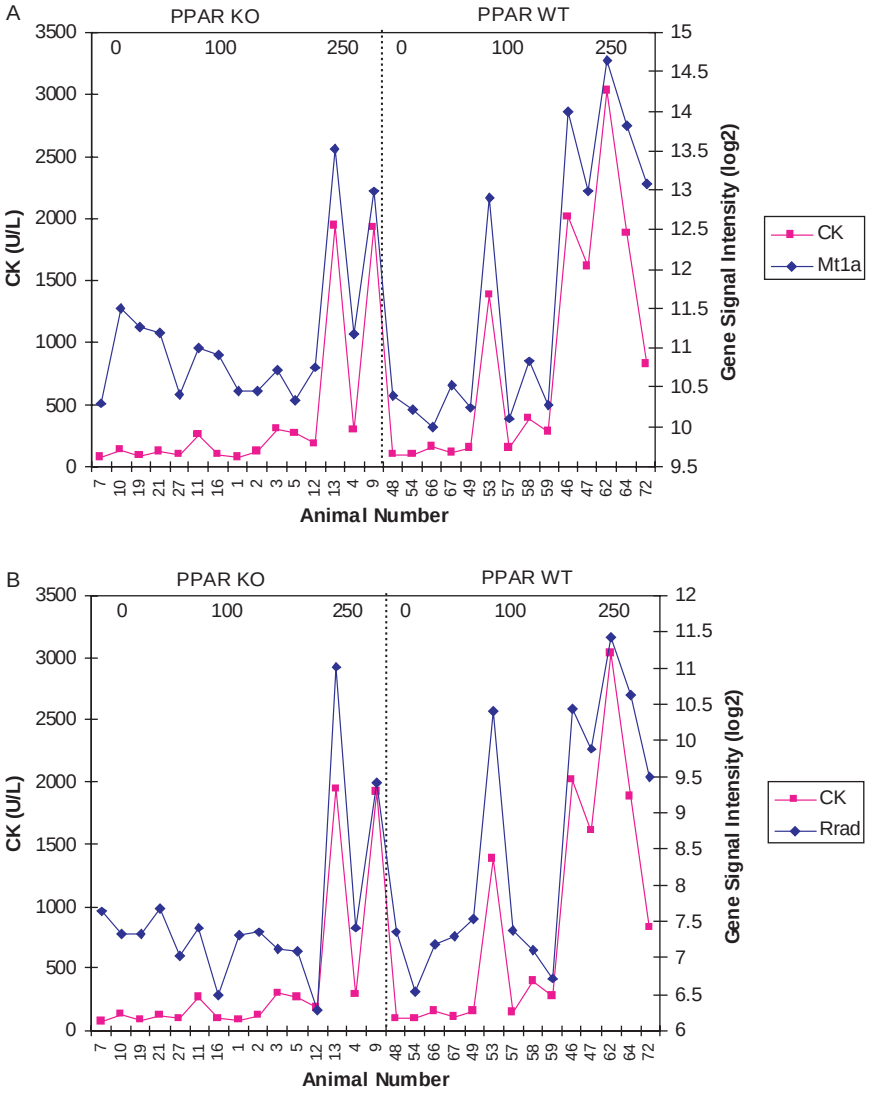

Figure 2. Serum creatine kinase (CK) vs *Mt1a* and *Rrad* gene expression from individual animals. CK values are plotted against quadriceps *Mt1a* (A) and *Rrad* (B) $\log_2$- transformed gene expression signals from each animal used in this report. *Mt1a* and *Rrad* were the two genes demonstrating the highest correlation with serum CK values, with correlation coefficients of 0.937 and 0.935, respectively. Animal numbers are arranged by genotype and dose from left to right, starting with PPAR knockout (KO) groups ($n = 5$, control, 100 mg kg^{-1} daily, 250 mg kg^{-1} daily GW610742X) followed by PPAR wild-type (WT) groups (control, 100 mg kg^{-1} daily, 250 mg kg^{-1} daily GW610742X). Animal no. 60 (WT control) was excluded from gene expression analysis as a technical outlier and was therefore excluded from the remainder of the analysis, resulting in an $n = 4$ for the WT control group. Mt1a, metallothionine 1a; Rrad, Ras-related associated with diabetes.

with GW610742X. Consequently, changes in serum CK, which reflect total muscle damage, and would not necessarily be expected to correlate exclusively with myopathic lesions in this muscle (i.e. a small amount of damage to larger muscles may mask CK signals of more extensive damage to a smaller muscle such as soleus).

		PPAR ko															Wt													
		Ctl					100 mkd					250 mkd					Ctl					100 mkd				250 mkd				
Gene	Probe Set	7	10	19	21	27	11	16	1	2	3	5	12	13	4	9	48	54	66	67	49	53	57	58	59	46	47	62	64	72
Mt1a	1371237_a_at	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	+	+	+	+
Rrad	1367862_at	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	+	+	+	+
Stat3	1370224_at	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	+	+	+	+
Socs3	1377092_at	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	+	+	+	+
Mid1ip1	1372091_at	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	+	+	+	+
Ankrd1	1373619_at	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	+	+	+	+
	Myopathy	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	+	+	+	+	+	+

Figure 3. Myopathy and marker gene expression in quadriceps muscle of mice. Quadriceps muscle gene expression and histopathology data are given for each animal evaluated in the study. Animal numbers are given along with genotype (PPARko = B6.129S2-*Ppara*tm1N12, Wt = C57/BL6) and treatment (Ctl = control, 100 = 100 mg kg^{-1} daily GW610742X, 250 = 250 mg kg^{-1} daily GW610742X). Average gene expression signal intensities for each control group were used to calculate mean and standard deviation (sigma) values. Gene expression was considered to be upregulated (+, shaded box) if the signal was greater than two-sigma from the control mean value. Since the background level of myopathy can be high in certain muscle groups, myopathy scores are presented as relative to background; a muscle was scored positive for PPAR-induced myopathy (+, shaded box) if lesions were at least one grade above the average control grade.

Affymetrix GeneChip® signal intensities of the six putative PPAR-myopathy marker genes were obtained from rat soleus muscle as described above. Average signal intensities for each control group were used to calculate mean and standard deviation (sigma) values on day 3, day 5 and day 7 after treatment with 100 mg kg^{-1} daily of GW610742X, a dose we have found to be approximately equipotent as that used in the mouse study for inducing myopathy (data not shown). Gene expression was considered to be upregulated if the signal from an individual animal was greater than

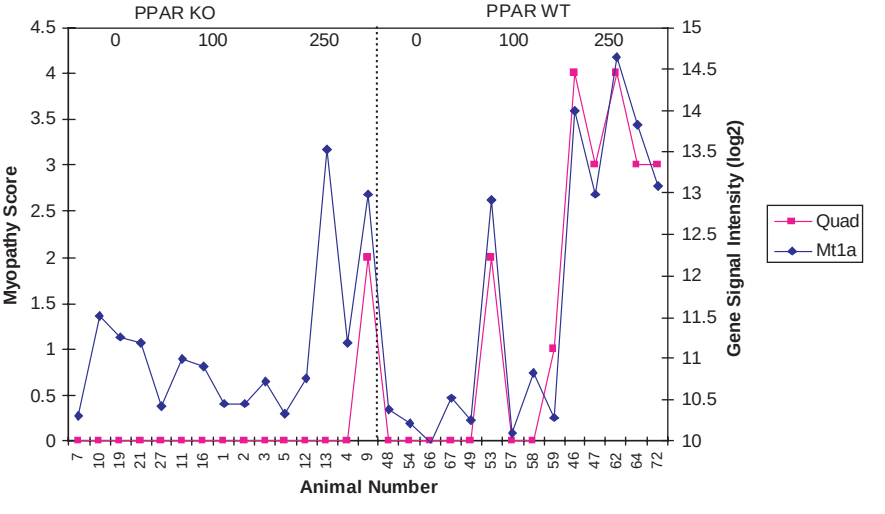

Figure 4. Quadriceps myopathy score vs *Mt1a g*ene expression. Myopathy scores are plotted against quadriceps *Mt1a* gene expression signals ($\log_2$ transformed) from each animal used in this report. Numeric values (myopathy scores) for severity grades of skeletal myopathy were assigned as follows: minimal = 1, mild = 2, moderate = 3, marked = 4. Animal numbers are arranged by genotype and dose from left to right, starting with PPAR knockout (KO) groups (n = 5, control, 100 mg kg^{-1} daily, 250 mg kg^{-1} daily GW610742X) followed by PPAR wild-type (WT) groups (control, 100 mg kg^{-1} daily, 250 mg kg^{-1} daily GW610742X). Animal no. 60 (WT control) was excluded from gene expression analysis as a technical outlier and was therefore excluded from the remainder of the analysis, resulting in an n = 4 for the WT control group. Quad, Quadriceps; Mt1a, metallothionine 1a.

		Day 3									Day 5										Day 7									
		Ctl					742X				Ctl					742X					Ctl					742X					
Gene	Probe Set	1	2	3	4	5	16	17	18	19	6	7	8	9	21	22	23	24	25	11	12	13	14	15	26	27	28	29	30
Mt1a	1371237_a_at	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	-
Rrad	1367862_at	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+		-	-	-	-	-	+	+	+	+	-
Stat3	1370224_at	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	-	-	-	-	+	+	-	-	-
Socs3	1377092_at	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	-
Mid1ip1	1372091_at	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Ankrd1	1373619_at	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
	Myopathy	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+	-

Figure 5. Myopathy versus marker expression in skeletal muscle of rats from GSK Study R41505. Gene expression and histopathology data are given for each animal evaluated in the study. Animal numbers are given along with treatment (Ctl = control, GW610742X = 100 mg/ kg^{-1} daily GW610742X) and study day. Average gene expression signal intensities for each control group were used to calculate mean and standard deviation (sigma) values for soleus muscle. Gene expression was considered to be upregulated (+, shaded box) if the signal was greater than two-sigma from the control mean value. Since the background level of myopathy can be high in certain muscle groups, myopathy scores are presented as relative to background; a muscle was scored positive for PPAR-induced myopathy (+, shaded box) if lesions were at least one grade above the average control grade.

two-sigma from the mean value of all controls. Since the background level of myopathy can be high in certain muscle groups, myopathy scores were assessed relative to background levels. Gene expression and relative myopathy scores in the soleus are presented in Figure 5.

There appears to be a good association between the expression of two of the six genes examined, *Mt1a* and *Socs3,* and myopathy in the soleus of individual rats, with all increases in expression of these genes coinciding with an increase in myopathy. There were two animals in which histopathological evidence of myopathy was noted but there was no increase in either *Mt1a* or *Socs3* expression: animal no. 19 (day 3, 100 mg kg daily GW610742X) and animal no. 15 (day 7, control), as shown in Figure 5. The myopathy lesions in animal no. 15 were not treatment related, and given the constitutive high background level of myopathy in the soleus muscle, animal no. 19 may also represent a non-treatment-related observation (neither CK nor asparte aminotransferase levels were elevated in this animal, data not shown). The absence of both marker gene upregulation and myopathy in animal no. 30 (day 7, 100 mg kg^{-1} daily GW610742X) is noteworthy in that it indicates that induction of *Mt1a* and *Socs3* in treated animals with myopathy is not directly associated with a PPAR-response element in these genes (classic PPAR responsive genes such as *Mte1* and *Ech1* were strongly upregulated in animal no. 30, data not shown).

In summary, Affymetrix GeneChip data from the quadriceps muscles from 29 individual mice were correlated with serum CK levels using the CORREL (correlation) function in Microsoft Excel. Six genes were identified which had correlation coefficients of ≥ 0.90. Proteins encoded by these six genes shared physiological functions relating to oxidative stress, inhibition of glycolysis and cytokine signalling; thus suggesting that oxidative stress and disregulation of energy balance may play a role in the pathogenesis of PPAR-induced myopathy.

Genes which were highly correlated with serum CK in wild-type and PPARα knockout mice showed good association with histopathological observations of myopathy in quadriceps of these animals. Expression of these genes was subsequently examined over a 7-day time course in skeletal muscle from individual Sprague Dawley rats treated with a myopathic dose of GW610742X. Two genes,

Mt1a and *Socs3*, were found to have a good association between increased mRNA levels and incidence of myopathy in the soleus. Eight of nine GW610742X-treated rats with myopathic lesions in the soleus also had increased mRNA for both *Mt1a* and *Socs3*, while there were no control animals in either study which had increased levels of these transcripts.

Results from this study demonstrate the utility of using correlation analysis of individual animal data for biomarker identification and mechanistic investigations. The ability to utilize data generated in one species and apply it to a different muscle type from a different species speaks to the translational potential of this approach. Compared with standard group mean comparison methods (*p*-value, fold-change), this approach reduces the number of animals needed to reach a valid result while also increasing the confidence in the findings.

References

Abel J, Ruiter N. 1989. Inhibition of hydroxyl-radical-generated DNA degradation by metallothionein. Toxicology Letters 47:191–196.

Anderson S P, Howroyd P, Liu J, Qian X, Bahnemann R, Swanson C, Kwak MK, Kensler TW, Corton JC. 2004. The transcriptional response to a peroxisome proliferator-activated receptor alpha agonist includes increased expression of proteome maintenance genes. Journal of Biological Chemistry 279:52390–52398.

Arany I, Megyesi JK, Nelkin BD, Safirstein RL. 2006. Kidney STAT3 attenuates EGFR-mediated ERK activation and cell survival during oxidant stress in mouse proximal tubular cells. Kidney International 70:669–674.

Bjorbaek C, Elmquist JK, Frantz JD, Shoelson SE, Flier JS. 1998. Identification of SOCS-3 as a potential mediator of central leptin resistance. Molecular Cell 1:619–625.

Bjorbaek C, El-Haschimi K, Frantz JD, Flier JS. 1999. The role of SOCS-3 in leptin signaling and leptin resistance. Journal of Biological Chemistry 274:30059–30065.

Chen C, Hennig GE, Whiteley HE, Corton JC, Manautou JE. 2000. Peroxisome proliferator-activated receptor alpha-null mice lack resistance to acetaminophen hepatotoxicity following clofibrate exposure. Toxicological Sciences 57:338–344.

Desvergne B, Michalik L, Wahli W. 2006. Transcriptional regulation of metabolism. Physiological Review 86:465–514.

Dhawan D, Goel A. 1995. Further evidence for zinc as a hepatoprotective agent in rat liver toxicity. Experimental & Molecular Pathology 63:110–117.

Emanuelli B, Peraldi P, Filloux C, Chavey C, Freidinger K, Hilton DJ, Hotamisligil GS, Van Obberghen EI. 2001. SOCS-3 inhibits insulin signaling and is up-regulated in response to tumor necrosis factor-alpha in the adipose tissue of obese mice. Journal of Biological Chemistry 276:47944–47949.

Farrell GC. 2005. Signalling links in the liver: Knitting SOCS with fat and inflammation. Journal of Hepatology 43:193–196.

Feige JN, Gelman L, Michalik L, Desvergne B, Wahli W. 2006. From molecular action to physiological outputs: peroxisome proliferator-activated receptors are nuclear receptors at the crossroads of key cellular functions. Progress in Lipid Research 45:120–159.

Fernandez V, Castillo I, Tapia G, Romanque P, Uribe-Echevarria S, Uribe M, Cartier-Ugarte D, Santander G, Vial MT, Videla LA. 2007. Thyroid hormone preconditioning: protection against ischemia-reperfusion liver injury in the rat. Hepatology 45:170–177.

Guri A, Hontecillas R, Bassaganya-Riera J. 2006. Peroxisome proliferator-activated receptors: bridging metabolic syndrome with molecular nutrition. Clinical Nutrition 25:871–885.

Hodel C. 2002. Myopathy and rhabdomyolysis with lipid-lowering drugs. Toxicology Letters 128:159–168.

Ilany J, Bilan PJ, Kapur S, Caldwell JS, Patti M-E, Marette A, Kahn CR. 2006. Overexpression of Rad in muscle worsens diet-induced insulin resistance and glucose intolerance and lowers plasma triglyceride level. Proceedings of the National Academy of Science USA 103:4481–4486.

Issemann I, Green S. 1990. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature 347:645–650.

Jeyaseelan R, Poizat C, Baker RK, Abdishoo S, Isterabadi LB, Lyons GE, Kedes L. 1997. A novel cardiac-restricted target for doxorubicin. CARP, a nuclear modulator of gene expression in cardiac progenitor cells and cardiomyocytes. Journal of Biological Chemistry 272:22800–22808.

Jiang LJ, Maret W, Vallee BL. 1998. The glutathione redox couple modulates zinc transfer from metallothionein to zinc-depleted sorbitol dehydrogenase. Proceedings of the National Academy of Science USA 95:3483–3488.

Kolbus A, Blazquez-Domingo M, Carotta S, Bakker W, Luedemann S, von Lindern M, Steinlein P, Beug H. 2003. Cooperative signaling between cytokine receptors and the glucocorticoid receptor in the expansion of erythroid progenitors: molecular analysis by expression profiling. Blood 102:3136–3146.

Laville M, Auboeuf D, Khalfallah Y, Vega N, Riou JP, Vidal H. 1996. Acute regulation by insulin of phosphatidylinositol-3-kinase, Rad, Glut 4 and lipoprotein lipase mRNA levels in human muscle. Journal of Clinical Investigation 98:43–49.

Li Y, Takemura G, Okada H, Miyata S, Maruyama R, Li L, Higuchi M, Minatoguchi S, Fujiwara T, Fujiwara H. 2006. Reduction of inflammatory cytokine expression and oxidative damage by erythropoietin in chronic heart failure. Cardiovascular Research 71:684–694.

Maret W. 1994. Oxidative metal release from metallothionein via zinc-thiol/disulfide interchange. Proceedings of the National Academy of Science USA 91:237–241.

Moller DE, Bjorbaek C, Vidal-Puig A. 1996. Candidate gene for insulin resistance. Diabetes Care 19:396–400.

Moyers JS, Bilan PJ, Reynet C, Kahn CR. 1996. Overexpression of Rad inhibits glucose uptake in cultured muscle and fat cells. Journal of Biological Chemistry 271:23111–23116.

Peraza MA, Burdick AD, Marin HE, Gonzalez FJ, Peters JM. 2006. The toxicology of ligands for peroxisome proliferator-activated receptors (PPAR). Toxicological Sciences 90:269–295.

Powell SR. 2000. The antioxidant properties of zinc. Journal of Nutrition 130:1447S–1454S.

Qing Y, Stark GR. 2004. Alternative activation of STAT1 and STAT3 in response to interferon-gamma. Journal of Biological Chemistry 279:41679–41685.

Quesada AR, Byrnes RW, Krezoski SO, Pettering DH. 1996. Direct reaction of H2O2 with sulfhydryl groups in HL-60 cells: zinc-metallothionein and other sites. Archives of Biochemistry & Biophysics 334:241–250.

Reynet C, Kahn CR. 1993. Rad: a member of the Ras family overexpressed in muscle of type II diabetic humans. Science 262:1441–1444.

Schafer SC, Wallerath T, Closs EI, Schmidt C, Schwartz PM, Forstermann U, Lehr HA. 2005. Dexamethasone suppresses eNOS and CAT-1 and induces oxidative stress in mouse resistance arterioles. American Journal of Physiology. Heart & Circulatory Physiology 2005 288:H436–H444.

Senn JJ, Klover PJ, Nowak IA, Zimmers TA, Koniaris LG, Furlanetto RW, Mooney RA. 2003. Suppressor of cytokine signaling-3 (SOCS-3), a potential mediator of interleukin-6-dependent insulin resistance in hepatocytes. Journal of Biological Chemistry 278:13740–13746.

Shankar K, Vaidya VS, Corton JC, Bucci TJ, Liu J, Waalkes MP, Mehendale HM. 2003. Activation of PPAR-alpha in streptozotocin-induced diabetes is essential for resistance against acetaminophen toxicity. Faseb Journal 17:1748–1750.

Szasz G, Gruber W, Brent E. 1976. Creative kinose in serum: 1. Determination of optimum reaction conditions. Clinical Chemistry 22:650–656.

Sznaidman ML, Haffner CD, Maloney PR, Fivush A, Chao E, Goreham D, Sierra ML, LeGrumelec C, Xu HE, Montana VG, et al. 2003. Novel selective small molecule agonists for peroxisome proliferator-activated receptor delta (PPARdelta) – synthesis and biological activity. Bioorganic & Medicinal Chemistry Letters 13:1517–1521.

Willson TM, Brown PJ, Sternbach DD, Henke BR. 2000. The PPARs: from orphan receptors to drug discovery. Journal of Medicinal Chemistry 43:527–50.

Witt CC, Ono Y, Puschmann E, McNabb M, Wu Y, Gotthardt M, Witt SH, Haak M, Labeit D, Gregorio CC, Sorimachi H, Granzier H, Labeit S. 2004. Induction and myofibrillar targeting of CARP, and suppression of the Nkx2.5 pathway in the MDM mouse with impaired titin-based signaling. Journal of Molecular Biology 336:145–154.