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White Paper

Development and Validation of an ELISA for the CK18-M30 Apoptosis Biomarker for NASH Drug Development

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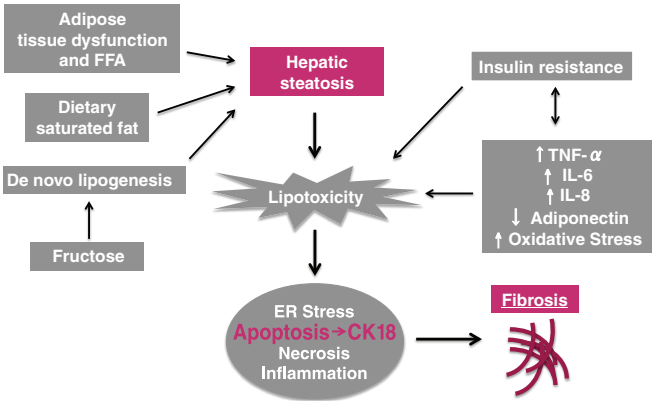
Introduction

Nonalcoholic fatty liver disease (NAFLD) is a spectrum of liver dysfunction associated with hepatic steatosis (fat accumulation), and nonalcoholic steatohepatitis (NASH) represents a more severe form of the disease resulting in a 10 year mortality rate of 60%. Approximately 80 million Americans and 1 billion worldwide are affected by NAFLD and 25% progress to NASH. In addition, NASH is currently the second leading indication for liver transplant. The rising incidence of NASH is expected to accelerate as the disease is strongly associated with diabetes and obesity, which have both reached epidemic proportions.

Currently, painful liver biopsy procedures are used to diagnose NASH and assess treatment response. Consequently, there is imperative need for the development of non-invasive soluble biomarkers for this indication.

CK18 is a Soluble Biomarker for NASH

Figure 1. Apoptosis mediates the progression to NASH fibrosis and is detected by the release of CK18 protein fragments



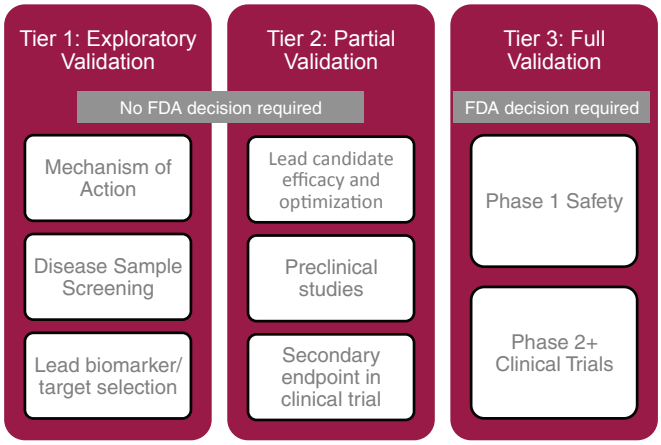
A soluble biomarker is a measurable factor found in bodily fluids (blood, urine, saliva, etc.) from an individual whose presence is indicative of a specific phenomenon, such as a disease, and can provide an alternative cost effective, non-invasive manner to monitor the efficacy of a therapeutic.

The prominent characteristic of NASH is hepatocyte cell death and fibrosis due to apoptosis. Early on in the apoptosis of hepatocytes, caspases (a form of proteases) are activated and cleave the protein keratin, and the resulting fragments are subsequently released into the blood. These fragments are known as CK18 or M30. Numerous studies show this caspase cleaved CK18 fragment is a sensitive and specific biomarker for NASH that rises with increasing severity of NASH and decreases with exercise therapy programs. This suggests that CK18 is a valuable soluble marker for diagnosis of NASH and a biomarker that can demonstrate the efficacy of NASH treatments.

Biomarker Validation

Most CK18 assays are for research use only and not suitable for regulatory submissions. Assays must be adapted to comply with 2013 FDA Bioanalytical Guidance and fit the context of use (COU) of the biomarker. We developed a three-tiered framework for validation of biomarkers that tailors the validation and assay adaptations to the COU of the biomarker. Tier 1 Exploratory Validation is appropriate when the COU is for internal decision making (e.g. novel biomarkers, understanding the drug mechanism, or selecting a lead candidate). Tier 2 Partial Validation is used for data that plays a supporting role in a regulatory claim (i.e. a secondary endpoint in clinical trials). This includes markers of inflammation and apoptosis such as CK18 protein expression which can be used in a regulatory filing to support primary endpoints such as improvement in fibrosis. A Tier 3 Full Validation is required for primary endpoint assays for a critical regulatory decision. We developed and validated a quantitative assay for CK18 to Tier 2 validation based on a context of use as a secondary endpoint in NASH clinical trials.

Figure 2. Flexible Biomarker Method Validation Tiers Based on Context of Use Optimize Cost, Speed, and Data Delivery During the Drug Development Spectrum



Method

We developed an ELISA for CK18 to support clinical trial sample analysis. Briefly, serum samples and enzyme-labeled antibody were pipetted onto microplates previously coated with an appropriate capture antibody. The wells were washed to remove any unbound sample material and antibody. A chromogenic substrate was added, resulting in development of the colored reaction product being directly proportional to the amount of CK18 present in the sample. The microplate was then analyzed using a colorimetric plate reader. Calibrator material was used to make 3 quality control samples (QCs) and an endogenous QC was established using pooled human serum.

Results

Assay Precision

CK18 uses a 4-parameter logistic regression weighted 1/Y2 over the analytical range 64.7 U/L – 964 U/L (1 U/L = 1.24 pM). Inter-batch precision (%CV) of quality control samples was equal to or less than 14.0.

Table 1. CK18 Inter-batch Precision

CK18	LLOQ QC U/L	Low QC U/L	Mid QC U/L	Endo QC U/L	High QC U/L	ULOQ QC U/L
Inter-BatchMean	64.7	138	378	115	648	964
Inter-Batch SD	6.86	11.1	26.0	16.0	50.8	71.5
Inter-Batch % CV	10.6	8.01	6.88	14.0	7.83	7.42
n	12	16	16	22	16	12

Matrix Effect

Matrix effect was evaluated in serum from both disease-free humans and serum from patients with the intended disease of the context of use of the biomarker (NASH). Serum was spiked with CK18 and the measured concentration of 10 lots was within ±20% of the expected concentration as shown in Table 2.

Table 2. No Observed Matrix Effect of CK18 in NASH Serum

		High Spike			
	Mean Basal Level	Nominal Amount Spiked	Expected Concentration	Measured Concentration	
Lot#	U/L	U/L	U/L	U/L	% Dev.
1	145	600	745	820	+10.1
2	107	600	707	673	-4.8
3	260	600	860	834	-3.0
4	286	600	886	889	+0.3
5	374	571	945	989	+4.7
6	215	600	815	783	-3.9
7	139	600	739	627	-15.2
8	163	600	763	752	-1.4
9	437	462	899	840	-6.6
10	238	600	838	820	-2.1

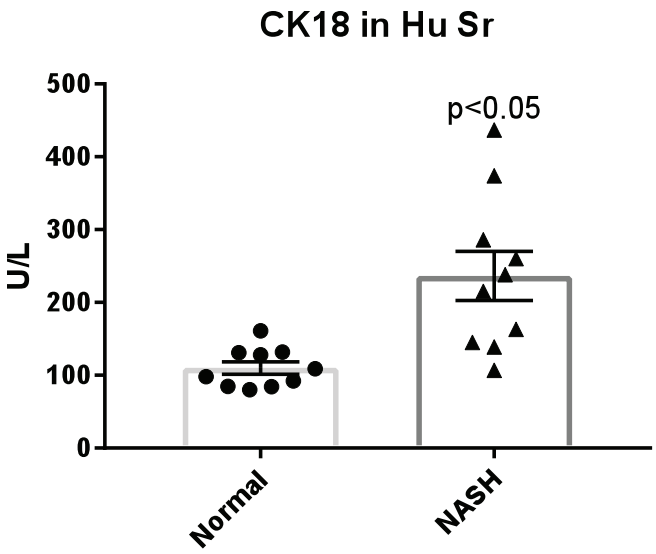
Parallelism

It is important to establish parallelism during bioanalytical validation of a biomarker to determine if the recombinant calibrator material behaves similarly to the endogenous biomarker. CK18 endogenous concentrations were not high enough to perform 4 dilutions within the assay range. Parallelism was thus evaluated by dilution integrity by spiking endogenous samples with a recombinant calibrator at a concentration higher than the upper limit of quantification. The measured concentration was within ±10% of the expected concentration for 3 dilutions. The data demonstrate that the recombinant CK18 is comparable to endogenous CK18 and suitable for use as a calibrator. Since the endogenous QC CK18 concentration was not high enough for parallelism analysis without spiking, this test will be repeated using study samples with high enough concentration of CK18 during in-study validation.

Table 3. Parallelism/ Dilution Integrity of CK18

	ENDO QC DF=10	2458 U/L ENDO QC DF=5	ENDO QC DF=4
	2570	2360	2460
	2490	2340	2520
	2470	2360	2410
	2590	2300	2540
	2620	2260	2550
	2590	2390	2430
Mean	2555	2335	2485
% CV	2.4	2.0	2.4
% Theoretical	103.9	95.0	101.1
n	6	6	6

Figure 3. CK18 Biomarker Levels in Normal and NASH Serum



CK18 in NASH Disease Matrix

Measurement of CK18 concentrations in normal and NASH serum (n=10/ group) showed a statistically significant increase in CK18 in NASH (un-paired t-test, p=0.0003). Furthermore, studies from other laboratories indicate the ELISA can distinguish between NAFLD and borderline NASH. This demonstrates the validated method is a valuable biomarker assay for NASH drug development.

Conclusion

Due to regulatory demand for quality biomarker data, a tiered validation framework can reduce drug development costs and time while improving assay quality and standardization. A method has been developed using this framework that allows for rapid, accurate, and reproducible measurement of CK18 in human serum samples. This method is suitable for measurement of NASH clinical samples where the context of use is exploratory or where CK18 is used as secondary clinical endpoint biomarker.

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