The Analytical and Clinical Validation of Caspase-Cleaved Keratin 18 Fragments, M30® as a Promising NAFLD Biomarker

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INTRODUCTION

There is a concerted effort to validate and implement *simple and noninvasive* approaches to diagnose nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) patients as well as identify potential participants for clinical studies for drug development.

Caspase-cleaved keratin 18 fragments (ccK18) detected by the M30® antibody, represent hepatic cell apoptosis and injury, correlate with hepatocyte ballooning (1), and have been proposed as inclusion criteria biomarkers for NAFLD clinical studies.

In a biopsy-proven NASH cohort, *Liebig et al.* suggested a M30® cutoff of 200 U/L could be applied to discriminate fibrosis stages (2).

The aim of the present study is to stratify an obese population by low (<200 U/L) and high (≥ 200 U/L) M30® concentrations to examine the utility of this M30® cutoff for NAFLD.

MATERIALS & METHODS

The ccK18 assay M30 Apoptosense® ELISA (DiaPharma) was analytically validated according to FDA guidelines for use in a clinical trial as a secondary endpoint (3). Specifically assay validation was performed to evaluate accuracy, precision, and parallelism.

Study participants were enrolled in a one-day screening event at Celerion clinics in Tempe, AZ and Lincoln, NE.

Eligible participants had a BMI >30 kg/m^2 and were 18-65 years of age. Participants were excluded from the screening if they had an electronic implantable device or were pregnant.

During the study visit, blood draw for serum biomarkers and clinical chemistry labs were collected. Liver fat (CAP) and liver stiffness (VCTE) were determined with FibroScan® (Echosens, France).

BIOANALYTICAL VALIDATION

Table 1: M30® Assay Meets Inter-Batch Assay Precision

M30®	LLOQ QC U/L	Low QC U/L	Mid QC U/L	High QC U/L	ULOQ QC U/L	Endo QC U/L
n	12	16	16	16	12	22
Inter-Batch Mean	64.7	138	378	648	964	115
Inter-Batch SD	6.86	11.1	26.0	50.8	71.5	16.0
Inter-Batch % CV	10.6	8.01	6.88	7.83	7.42	14.0

CV, correlation of variance; Endo, endogenous control; LLOQ, lower level of quantitation; QC, quality control; SD, standard deviation; ULOQ, upper level of quantitation

M30® uses a 4-parameter logistic regression weighted 1/Y^2 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.

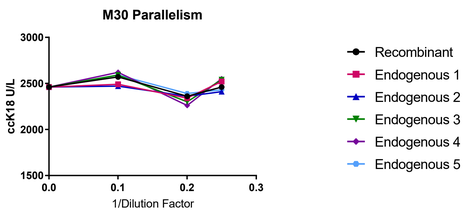
Table 2: No Observed Matrix Effect of ccK18 in NASH Serum

Serum from both disease-free humans and NASH patients was spiked with ccK18 to evaluate a matrix effect. The concentration of 10 lots was within ±20% of the expected concentration.

		High Spike			
	Mean Basal ccK18 Concentration	Nominal Amount Spiked	Expected ccK18 Concentration	Measured ccK18 Concentration	
Lot#	U/L	U/L	U/L	U/L	% Deviation
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

Figure 1. Parallelism of M30® Assay

Parallelism examines if the recombinant calibrator material behaves similarly to the endogenous biomarker. The measured concentration was within ±10% of the expected concentration for 3 dilutions.



CLINICAL EVALUATION

Table 2: Subject Characteristics and Clinical Chemistry Grouped by M30® Cutoff

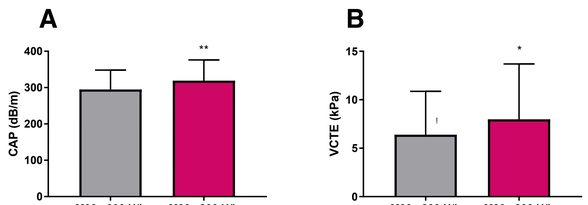
Parameter	M30<200 (n=138)	M30≥200 (n=59)	Entire Cohort (n=197)
Age (years)	44.5±10.4	41.8±12.0	43.6±11.0
Male/Female	48M/90F	22M/37F	70M/127F
BMI (kg/m^2)	35.9±5.4	36.9±5.8	36.2±5.5
ALT (U/L)	23.4±10.8	41.4±25.3***	28.8±18.4
AST (U/L)	20.3±6.5	31.4±18.5***	23.6±12.5
Glucose (mg/dL)	103.5±26.1	117.1±49.9*	107.5±35.4
HbA1c (%) [a]	5.6±0.4	6.1±1.2**	5.7±0.8
Triglyceride (mg/dL)	132.9±92.4	178.1±83.2**	146.5±91.9
Cholesterol (mg/dL)	187.8±31.7	192.7±38.4	189.3±33.8
FibroScan-AST (FAST)	0.13±0.13	0.29±0.25***	0.18±0.19
FIB4	0.77±0.34	0.87±0.70	0.80±0.47
Hepatic Steatosis Index (HSI)	46.3±7.0	49.3±7.2**	47.2±7.2
M65® (U/L)	221.9±115.7	488.4±342.9***	302.1±243.6
M30® (U/L)	115.6±48.8	328.1±162.5***	179.3±137.8

*a – HbA1c was measured on a subset of samples, n=52 for M30<200, n=29 for M30≥ 200, n=81 Entire Cohort. Results presented as mean±SD where * p<0.05, **p<0.01, ***p<0.001 by t-test.*

The M30®≥200 group displayed statistically significant higher levels of ALT (+44%), AST (+35%), glucose (+12%), HbA1c (+8%) and TG (+25%) compared to subjects with M30® below the cutoff.

NAFLD/NASH panels such as Hepatic Steatosis Index (HSI) (4) and FibroScan-AST (FAST) (5) were also statistically different between the two groups, whereas FIB4 (6) was not.

Figure 2. M30®> 200 is Associated with Higher Liver Fat and Stiffness



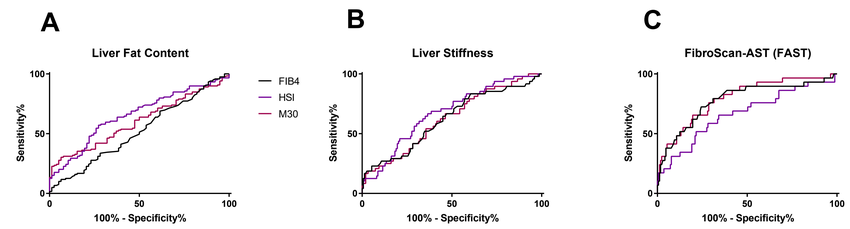
*Results presented as mean±SD where * p<0.05, **p<0.01 by t-test.*

Liver fat and stiffness were examined with noninvasive FibroScan technology. Participants with high M30® demonstrated elevated CAP (+8%) and VCTE (+20%) scores.

Average CAP and VCTE values for the entire cohort were 302.0±55.6 dB/m and 6.9±4.9 kPa respectively, and the X Large probe was used on 41% of participants.

CLINICAL PERFORMANCE

Figure 3. M30® Outperformed FIB4 to Predict Steatosis by CAP



M30® clinical performance was compared to FIB4 and HSI to predict liver fat defined as CAP≥ 300 dB/m and liver stiffness as VCTE≥7kPa. Performance was determined by the area-under the receiver operator curve (AUROC) [95% CI].

The AUROC for liver fat was higher for M30® (AUROC=0.64 [0.57, 0.72]) than for FIB4 9AUROC=0.50 [0.42, 0.59]) yet comparable to HSI (AUROC=0.69 [0.62, 0.77]), suggesting good ability to predict steatosis.

Both M30® and FIB4 demonstrated similar performance to predict liver stiffness, M30 AUROC=0.62 [0.53, 0.71] & FIB4 AUROC=0.62 [0.52, 0.71]. HSI was slightly higher (AUROC=0.67 [0.59, 0.76]).

The FAST cutoff of 0.35 has been proposed to rule out NASH patients that do not meet NAS≥4 and Fibrosis stage ≥2 criteria (5). Here, M30® and FIB4 performed well against this score with AUROC of 0.80 [0.71, 0.87] and 0.78 [0.68, 0.88] respectively.

Table 3. M30® Cutoff Demonstrated Good Specificity for FibroScan Scores

Analysis	CAP	VCTE	FAST
Sensitivity (%)	40% [31, 50]	38% [25, 52]	66% [47, 80]
Specificity (%)	81% [72, 88]	72% [65, 79]	76% [69, 82]
Positive Predictive Value (%)	69% [57,80]	31% [20, 43]	32% [22, 45]
Negative Predictive Value (%)	56% [47,64]	78% [71, 84]	93% [87, 96]

CONCLUSIONS

The M30® assay demonstrated good analytical accuracy, precision, and parallelism with endogenous analyte, indicating the assay is fit-for the purpose of a secondary endpoint in a clinical trial.

In an obese cohort, the M30® cutoff value of 200 U/L was associated with elevated liver fat and stiffness as well as AST, ALT, glucose, and triglycerides, suggesting metabolic disturbance in this group and possible NAFLD.

A main limitation of the study is that we were unable to examine M30® performance against liver biopsy or more sophisticated noninvasive technology such as magnetic resonance imaging or elastography.

When compared to other NAFLD/NASH panels, M30® outperformed FIB4 and was comparable to HSI for liver fat. Previous studies found M30® to be a good indicator of hepatic fibrosis in NASH patients (2). Using surrogate assessments of fibrosis such as VCTE and FAST, M30® performed similarly to FIB4.

The M30® cutoff also demonstrated good specificity and negative predictive value for VCTE and FAST.

As a screening tool, M30® cutoff may help exclude participants that do not show signs of the disease. Overall, as an analytically validated assay, M30® is a promising noninvasive NAFLD biomarker.

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