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## Intra-individual fasting versus postprandial variation of biochemical markers of liver fibrosis (FibroTest) and activity (ActiTest)

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### Abstract

**Background:** Biochemical marker combinations, including  $\alpha_2$ -macroglobulin, haptoglobin, apolipoprotein A1,  $\gamma$ -glutamyl transpeptidase, and total bilirubin (all part of FibroTest) plus alanine aminotransferase (all part of ActiTest), are being developed as alternatives to liver biopsy in patients with chronic hepatitis C and other various chronic liver diseases. Considering this premise, the primary aim of this study was to assess the impact of meal intake on FibroTest and ActiTest results. Such studies are very important for patients, as many clinical errors have been related to the absence of baseline evidence.

**Results:** Intra-individual variation was assessed for the 6 above components and for FibroTest and ActiTest, by measuring time dependent variations before and one hour after a standard meal in 64 subjects. These consisted of 29 healthy volunteers and 35 patients with chronic liver diseases. Meal intake had no significant impact on any of the six components, or on FibroTest or ActiTest, as assessed by repeated measure variance analyses (ANOVA all  $p > 0.90$ ); the Spearman correlation coefficient ranged from 0.87 (total bilirubin) to 0.995 ( $\gamma$ -glutamyl transpeptidase). The coefficients of variation (CV) between fasting and postprandial measurements fluctuated for the six components from 0.09 (apolipoprotein A1) to 0.14 ( $\alpha_2$ -macroglobulin), and from 0.09 for FibroTest to 0.13 for ActiTest. In contrast, meal intake had a significant impact on triglycerides (ANOVA  $p = 0.01$ , CV = 0.65) and glucose (ANOVA  $p = 0.04$ , CV = 0.31). As for the prediction of liver injury, the concordance between fasting and postprandial predicted histological stages and grades was almost perfect, both for FibroTest (kappa = 0.91,  $p < 0.001$ ) and ActiTest (kappa = 0.80,  $p < 0.001$ ).

**Conclusions:** The intra-individual variation of biochemical markers was low, and it was shown that measurements of FibroTest, ActiTest and their components are not significantly modified by meal intake. This fact makes the screening of patients at risk of chronic liver diseases more convenient.

## Background

One of the major clinical problems facing the medical community is how to best evaluate and manage the increasing numbers of patients with chronic liver diseases, including patients infected with hepatitis C virus (HCV) [1]. According to the latest consensus conferences, liver biopsy is still recommended in most patients [2,3]. However, recent studies strongly suggest that due to the limitations [4-6] and risks of biopsy [7], as well as the improvement of the diagnostic accuracy of biochemical markers [8,9], liver biopsy should no longer be considered mandatory.

Liver biopsy has three major limitations: the risk of adverse events [7], sampling error [4-6], and inter- and intra- pathologist variability [10,11]. An overview of published studies summarizes the risks of liver biopsy as pain (around 30%), severe adverse events (3/1000) and death (3/10 000) [7]. Sampling variation is the major cause of variability [4-6]. Bedossa et al. [6] observed very high coefficients of variation (55%) and high discordance rates (35%) for fibrosis staging in biopsies measuring 15 mm in length. The variability significantly improved in biopsies with 25 mm in length, but it was still very high, with a 45% coefficient of variation and 25% discordance rate [6].

Among the non-invasive alternatives to liver biopsy [9], numerous studies have demonstrated the predictive value of two combinations of simple serum biochemical markers in patients infected with HCV and HBV: FibroTest (FT) (Biopredictive, Houilles, France) for the assessment of fibrosis, and ActiTest (AT) (Biopredictive, Houilles, France) for the assessment of necroinflammatory activity [8,12-22]. Similar results have not been obtained by other diagnostic tests [9,12-14,16-22].

One concern regarding of biochemical panels is the variability due to the different components [23,24]. For FT, AT, and their six components, the inter- and intra laboratory variability has been shown to be acceptable without significant consequence on predictive values when pre-analytical and analytical recommendations were applied [25,26].

Because of the large number of patients who could benefit from these markers, it would be practical if fasting were not required for the determinations. Thus far, very few studies have looked at the impact of meal intake on liver tests, and none have been done specifically on the fibrosis markers [27-34].

The aim of this study was to assess the impact of meal intake on FibroTest and ActiTest results by measuring time dependent variations before and one hour after a standard

meal in 64 subjects. A secondary goal was to gain a better understanding of the relationship of liver function tests with fasting and meals.

## Results

A total of 65 subjects were screened between November 21<sup>st</sup> and December 19<sup>th</sup> 2003. These consisted of 35 patients and 30 apparently healthy volunteers. One volunteer did not come for the postprandial measurements and was therefore excluded. The characteristics of the 64 included subjects are given in Table 1. The only significant difference between healthy volunteers and patients characteristics, not directly related to liver disease, was the younger age of the former.

The analysis of inter- and intra-individual variability according to meal intake is given in Table 2 and data are quoted for all 64 subjects. Meal intake had no significant impact on any of the six biochemical components, nor on FibroTest or ActiTest, as assessed by repeated measure variance analysis; the Spearman correlation coefficient ranged from 0.87 (total bilirubin) to 0.995 ( $\gamma$ -glutamyl transpeptidase). The coefficients of variation between fasting and postprandial measurements fluctuated, for the six components, from 0.09 (apolipoprotein A1) to 0.14 ( $\alpha_2$ -macroglobulin), and 0.09 for FibroTest and 0.13 for ActiTest. In contrast, meal intake had a significant impact on triglycerides and glucose.

As for the prediction of liver injury, the concordance between fasting and postprandial predicted histological fibrosis stages and activity grades was almost perfect for both FibroTest and ActiTest (Tables 3,4).

When each individual variation was analyzed, very few variations were observed. No FT varied more than 0.10 points. No factors were found to be associated in multivariate analysis (Table 5). Only one AT result varied more than 0.10 decimals, from 0.34 to 0.52. This was in an apparently healthy volunteer with a small increase of fasting ALT (75 IU/l) and an increase in postprandial ALT (115 IU/l). Fasting and postprandial FT's were normal. The total calories of the meal were 790 kcal, of which 41% were lipids and 42% carbohydrates. A complete medical analysis was performed (viral markers, auto-immune markers, ultrasonography, thyroid hormones) which found no cause of chronic liver disease. The only abnormal findings were a small increase in body mass index (26.8 kg/m<sup>2</sup>), long-term medication use of the contraceptive pill and the ingestion of a NSAID (ibuprofen) for pelvic pain 48 hours before sampling.

There was no significant association between patients' characteristics and the dietary components of the meal (Table 5). One patient did not fill out the dietary ques-

**Table 1: Characteristics of included apparently healthy volunteers and patients**

	Apparently healthy volunteers	Patients
<b>Number of patients</b>	29	35
<b>Characteristics</b>		
Age (years) [mean (SD)]***	36 (8)	52 (15)
Male [n (%)]	12 (41)	22 (63)
Female [n (%)]	17 (59)	13 (37)
<b>Ethnic Origin</b>		
Caucasian [n (%)]	19 (65)	20 (60)
Non Caucasian [n (%)]	10 (35)	15 (40)
Black/Asian/Other (n)	5/1/4	5/3/7
Weight (kg) [mean (SD)]	68.2 (10.8)	69.7 (14.8)
Height [mean (SD)]	170 (9.5)	169 (7.6)
Body Mass Index (kg/m <sup>2</sup> ) [mean (SD)]	23.6 (3.4)	24.4 (5.1)
<b>Alcohol in g per day [n (%)]</b>		
0	21 (72)	21 (64)
0–50	8 (28)	8 (24)
Over 50	0 (0)	4 (12)
<b>Drug [n (%)]</b>	8 (28)	29 (83)
Anti-diabetic (n)	0	7
Oral contraception (n)	2	7
Other (n)	6	15
<b>Associated Disease</b>	0	35
Alcoholic Liver Disease (%)	0	10 (29)
Hepatitis C (%)	0	6 (17)
Hepatitis B (%)	0	4 (11)
Human Immunodeficiency Virus Infection (%)	0	1 (3)
Other (%)	0	14 (40)
<b>Histological prediction fasting</b>		
<b>Necrotico-inflammatory activity</b>		
No or minimal activity (%)	28	14
Moderate or severe activity (%)	1	21
<b>Fibrosis</b>		
No or minimal fibrosis (%)	28	14
Moderate (%)	1	5
Extensive fibrosis or cirrhosis (%)	0	16
<b>Baseline Biochemistry fasting</b>		
ALT (IU/L) [mean (SD)]***	23.7 (16.3)	67.4 (53.8)
Total Bilirubin (μmoles/L) [mean (SD)]	13.9 (7.6)	27.1 (31.7)
GGT U/L (7–32 female) (11–49 male) [mean (SD)]***	21 (15.9)	147 (149)
Alpha2 macroglobulin g/L (1.6–4.0) [mean (SD)]	1.77 (0.43)	2.15 (0.78)
ApoA1 g/L (1.2–1.7) [mean (SD)]*	1.56 (0.24)	1.26 (0.55)
Haptoglobin g/l (0.35–2.00) [mean (SD)]	1.06 (0.35)	0.86 (0.66)
Conjugated bilirubin (μmoles/L) [mean (SD)]*	3.25 (1.5)	28.9 (22.5)
Gilbert possible (%)	7	0
Fibrosis Index (range 0.00–1.00) [mean (SD)]***	0.13 (0.08)	0.55 (0.33)
Activity Index (range 0.00–1.00) [mean (SD)]***	0.09 (0.1)	0.42 (0.24)
AST (IU/L) [mean (SD)]***	26 (10)	60 (34)
Triglycerides (umol/L) [mean (SD)]	1.0 (0.76)	1.7 (2.6)
Cholesterol (umol/L) [mean (SD)]	4.8 (1.2)	4.5 (1.8)
Blood glucose (umol/L) [mean (SD)]**	4.2 (0.56)	5.9 (3.12)
<b>Dietary factors</b>		
Total calories (kcal)	788	774
Lipids (%)	38	35
Carbohydrates (%)	45	44
Proteins (%)	15	18
Fibers (g)	6	8

\*Significant difference between groups  $P < 0.05$ ; \*\*Significant difference between groups  $P < 0.001$ ; \*\*\*Significant difference between groups  $P < 0.0001$ .

**Table 2: Inter and intra-individual variability of FibroTest and ActiTest components and two positive controls (glucose and triglycerides) according to fasting**

	A2M g/L	HPT g/L	APOA I g/L	BT μmol/L	GGT U/L	ALT U/L	Fibro Test	Acti Test	Glucose	Triglycerides
<b>Mean (SD)</b>										
Fasting	1.98 (0.67)	0.95 (0.55)	1.40 (0.46)	21 (25)	90 (127)	48 (47)	0.36 (0.33)	0.27 (0.25)	5.09 (2.43)	1.38 (1.97)
Postprandial	1.99 (0.64)	0.96 (0.53)	1.40 (0.46)	21 (24)	88 (123)	47 (42)	0.36 (0.33)	0.27 (0.25)	6.17 (3.15)	1.88 (1.94)
<b>Spearman Coefficient</b>	0.96	0.98	0.94	0.87	0.995	0.98	0.98	0.98	0.45	0.75
<b>ANOVA p value</b>	0.92	0.93	0.98	0.99	0.98	0.94	0.98	0.94	0.04	0.01
<b>Coefficient of Variation (log)*</b>										
Inter-individual Fasting	0.34 (0.50)	0.58 (- 2.85)	0.33	1.17 (0.28)	1.41 (0.30)	0.98 (0.23)	0.91	0.27	0.48	1.43
Inter-individual Postprandial	0.32 (0.48)	0.55 (- 2.93)	0.33	1.15 (0.29)	1.40 (0.30)	0.90 (0.22)	0.90	0.27	0.51	1.03
Intra-individual	0.10	0.13 (-	0.09	0.25	0.14	0.18	0.09	0.13	0.31	0.65
Regression **	(0.14)	0.41)		(0.10)	(0.02)	(0.04)				

Data are quoted for all 64 subjects. \* As all components except apolipoprotein AI are computed using the logarithmic transformation in FibroTest and ActiTest formulas, the coefficient of variation was also assessed in log for these five components. \*\* Represents the intra-individual variability of the linear regression between fasting and postprandial values.

**Table 3: Fibrosis stages – Concordance between fibrosis stages and predictions using fasting or postprandial assessments**

Postprandial	Fasting		
	No or minimal Fibrosis	Moderate Fibrosis	Severe Fibrosis
No or minimal Fibrosis	39	0	0
Moderate Fibrosis	3	6	0
Severe Fibrosis	0	0	16

Kappa statistic = 0.91 (SD = 0.76); p < 0.001; almost perfect concordance.

**Table 4: Activity stages – Concordance between Activity grades predictions using fasting or postprandial assessments**

Postprandial	Fasting		
	No or minimal Activity	Moderate Activity	Severe Activity
No or minimal Activity	51	1	0
Moderate Activity	2	3	1
Severe Activity	0	0	6

Kappa statistic = 0.80 (SD = 0.77); p < 0.001; almost perfect concordance.

**Table 5: Association between postprandial variations of FibroTest-ActiTest and dietary factors**

	FibroTest	ActiTest
	Spearman Coefficient (significance p)	
<b>Characteristics</b>		
Age	+0.03 (0.80)	-0.11 (0.37)
Body Mass Index	-0.04 (0.73)	-0.03 (0.83)
<b>Meal components</b>		
Total Calories	-0.11 (0.38)	-0.02 (0.87)
Lipids	-0.11 (0.38)	-0.12 (0.35)
Carbohydrates	-0.24 (0.06)	-0.02 (0.87)
Fiber	+0.02 (0.90)	-0.14 (0.26)

tionnaire and he was consequently non-included in the dietary analysis.

### Discussion

This study demonstrated that a standard meal does not induce significant changes in the components' measurements, nor in the FT and AT values and predictions of histological lesions. This finding could facilitate the screening of liver injury in different chronic liver diseases, such as chronic hepatitis C [12-19] and B [20], alcoholic liver [35] or non-alcoholic steatosis [36], by replacing liver biopsy with biochemical markers. FT and AT cannot replace liver biopsy of sufficient quality (40 mm length) in clinical research or as a second line diagnostic tool for difficult cases in clinical practice. However, these markers should replace liver biopsy for routine estimation of liver injury in chronic liver diseases. The variability of the components and of FT-AT has been already studied for the pre-analytical and analytical steps [26], as well as for the inter-laboratory variability [25].

The analytical imprecision of the component results had only a slight impact on the FibroTest and ActiTest values. Intra-laboratory variability of fibrosis and activity indices was minor, ensuring the validation of clinically relevant diagnostic thresholds for significant fibrosis and activity. It was concluded that FibroTest and ActiTest component measurements must be performed on fresh serum or that which had been stored at -80°C. We also observed that the intra- and interseries imprecision of each parameter assay and of FT-AT were acceptable in the utilized analytical system, with the CV at about 5%. The upper CV of the ActiTest results (9.9%) in interseries imprecision was related to the lack of accuracy of the low normal ALT activity measurement itself. The logarithmic expression of results in the algorithm used for FibroTest and ActiTest calculations minimizes the influence of analytical imprecision, in particular for GGT, ALT and total

bilirubin. Variability in the same patient of 2 FT and AT results at an interval of 4 days was also acceptable in spite of isolated wider variations in components results [26]. The influence of gender was observed for all parameters except haptoglobin. ALT activity, as previously described, GGT activity,  $\alpha_2$ -macroglobulin, and haptoglobin levels were influenced by body mass index [26].

Curiously, very few studies have been published on the fasting or non-fasting variability of the 6 FT-AT components. Concentrations of 27 commonly estimated serum constituents were measured in blood sampled from 20 apparently healthy volunteers at 08:30, 12:30, and 16:30 hours on four consecutive days (at weekly intervals). Time-dependent statistically significant variations were observed for bilirubin, triglycerides, total protein, and albumin. An interesting finding for  $\alpha_2$  macroglobulin and haptoglobin, two main non-albumin proteins included in FT-AT, was that the variation of total protein was related to albumin variation, with higher concentrations in the morning than in the afternoon [27]. Patterns of bilirubin variation were variable, but there was a tendency for it to decrease in the afternoon samples. No statistically significant diurnal or weekly variation was observed for ALT,  $\alpha_2$ -macroglobulin, immunoglobulins (IgA, IgM, IgG), or cholesterol [27].

In the present study, the intra-individual variability was very low for total bilirubin, and for GGT and ALT serum activities (when expressed as logarithmic values). The mean variability observed was even lower than that observed previously between 2 measurements at 4 day intervals [26]. In only one case was there a difference in ALT, which was 43 IU/l more in the postprandial period, being a 60% increase in activity. This induced a difference in the ActiTest of 0.18.

We observed a non-significant intra-individual variability related to meals for  $\alpha_2$ -macroglobulin. Morrison et al. had already observed this [27], and Narita et al. also found that fractional clearance of  $\alpha_2$ -macroglobulin was not affected by protein meals as compared to smaller plasma inflammation proteins, such as ceruloplasmin or  $\gamma$ -globulins in healthy subjects [37].

No particular study has looked at postprandial variations of haptoglobin. As haptoglobin is associated with interleukin-6 and tumor-necrosis factor, it is possible that a modification of these cytokines could change the serum concentration. It is reassuring that a short-term isoenergetic, very low carbohydrate diet did not affect markers of inflammation, including interleukin-6, despite a significant decrease of fasting and postprandial triglycerides and an increase of HDL-cholesterol [31]. Keeping in line with these results, Morrison et al. also observed that non-albumin proteins (which include haptoglobin) were not modified after meals [27].

Apolipoprotein A1 variations have been studied in patients with hyperlipemia and cardiovascular diseases [30-34]. Mutation of the apolipoprotein A1 gene affects the LDL-cholesterol response to diet via mechanisms involving postprandial lipoprotein cholesterol metabolism [34]. There was a significant postprandial decrease in plasma cholesterol, LDL cholesterol, and apolipoprotein B in G/G subjects but not in G/A subjects. No significant genotype effects were detected for apolipoprotein A1 and HDL-cholesterol concentrations. [30,34] In the present study the variability related to meal intake was also much greater for triglycerides (Table 2) than for apolipoprotein A1.

Lipoprotein profiles change markedly during cardiac catheterisation [38], with total cholesterol triglycerides and apolipoprotein A1 serum concentrations decreasing significantly from the baseline concentrations. These changes were probably related to the activation of lipoprotein lipase due to heparin injection [39].

In the present study, the dietary characteristics of the meal were not associated with significant post-prandial variations. However, as the median total calories of the meal was 782 kilo-calories with a maximum of 1340 kcal, it is prudent to recommend a meal of around 800 kcal.

## Conclusions

This study shows that the biochemical markers of liver fibrosis (FibroTest) and activity (ActiTest) is low, and that these two tests can be performed in postprandial periods in order to facilitate the management of patients with chronic liver diseases.

## Methods

### Subjects

Two groups of subjects were included. The first was a group of apparently healthy volunteers without known liver disease. The second were patients hospitalized in our Department of Hepato-Gastroenterology due to chronic liver disease. Subjects with a history of gastric surgery were excluded, as were subjects unwilling to eat their usual lunch meal and subjects who refused to sign the informed consent.

### Questionnaire

A questionnaire with 17 items was filled out which included clinical characteristics and the details of the lunch in term of calories, fat, protein and carbohydrate composition according to Prodiat software (Nutrilog, Versailles, France) (Table 1).

### Design of the study

After giving consent for the study, subjects had one blood sample drawn between 7 and 10 a.m. after 10 hours of night fasting, and on the same day a postprandial blood sample was drawn 1 hour after the end of the usual meal. Blood samples were obtained from patients by venous puncture and collected in 5 ml glass tubes without anticoagulant. Serum samples were separated after coagulation, by centrifugation. The centrifugation conditions conformed to the recommendations of the Vacutainer tube manufacturers (Becton Dickinson Franklin Lakes NJ, USA). 500  $\mu$ l was sufficient for the 6 parameters assays.

### Biochemical markers

We used the previously validated FT-AT [8,12-22]. FT combines the following five markers, all independently related to fibrosis, as well as age and gender:  $\alpha_2$ -macroglobulin, haptoglobin,  $\gamma$ -glutamyl transpeptidase (GGT EC: 2.3.2.2), total bilirubin, and apolipoprotein A1. AT combines the same five markers plus alanine amino transferase (ALT EC: 2.6. 1. 2). The results of FT-AT can range from 0.00 to 1.00, with a conversion to METAVIR stages and grades calculated from median scores and 95% confidence intervals, which had been previously observed in 1,270 patients and 300 healthy blood donors [8,12,26,40]. GGT, ALT and total bilirubin were measured by a Hitachi Modular DP automat from Roche Diagnostics (Mannheim, Germany), using the manufacturer reagents. ALT was assessed according to the standard method recommended by the IFCC (International Federation of Clinical Chemistry), using pyridoxal phosphate as the activator [41]. GGT activity was assessed according to the Szasz method, standardized against the original method published by Persijn and Van der Slik [42], and total bilirubin by a diazoreaction according to the Wahlefeld method [43]. The assays for these three parameters were calibrated with a CFAS (calibrator for automated systems

from Roche Diagnostics). ALT enzymatic activity of the calibrator was determined with the reference method using the molar absorption coefficient ( $\epsilon$ ) measurement of NADPH, measured at 339 nm. The calibrator GGT activity was determined with the Szasz reference method using the coefficient  $\epsilon$  measurement of amino-5 nitro-2 benzoate at 410 nm. The calibrator bilirubin concentration was determined using the Standard Reference Material (SRM) 909b, and assessed according to the Doumas reference method [44].

The  $\alpha_2$ -macroglobulin, apolipoprotein A1, and haptoglobin were measured using a Modular analyzer (BNII, Dade Behring; Marburg, Germany). All CV assays were lower than 6%. Reagents used for the assays were standardized against the International Certified Reference Material 470 (CRM 470) for  $\alpha_2$ -macroglobulin and haptoglobin, and against the reference material of the World Health Organization – International Federation of Clinical Chemistry SP1-01 (WHO- IFCC SPI-01) for apolipoprotein A1 [45,46]. Glucose, triglycerides and cholesterol were measured by Hitachi Modular.

#### Statistical methods

Intra-individual variability according to fasting and postprandial measurements was assessed by repeated variance analysis, the Spearman rank coefficient of correlation and the coefficient of variation (standard variation divided by the mean). The coefficient of variation was calculated for the fasting, the postprandial values and the intra-individual regression analysis. Calculations were made with regular values and used logarithmic transformation for the 5 components expressed as a log value in the FibroTest and ActiTest algorithm. The coefficient of variation for the linear regression was computed by dividing the square root of the mean square error of the linear correlation coefficient (between fasting and postprandial values) by the mean of the fasting value [47].

Two serum biochemical parameters, glucose and triglycerides, which are well known to increase after meals, were used as positive controls.

The possible impact of age, body mass index, total meal calories, and respective calories due to carbohydrates and lipids on postprandial variation was assessed by the Spearman coefficient of correlation.

Analysis by kappa statistics was performed to assess the degree of concordance between predicted fibrosis stages (three classes using the METAVIR scoring system: no or portal fibrosis; few septa; many septa or cirrhosis) and grades (three classes using the METAVIR scoring system: no or minimal necrosis; moderate necrosis; severe necrosis) [10,11]. The strength of agreement was considered

slight for values between 0 and 0.19, fair for values between 0.20 and 0.39, moderate for values between 0.40 and 0.59, substantial for values between 0.60 and 0.79, and almost perfect if kappa values were greater than 0.80 [48,49]. All statistical analyses were performed with Number Cruncher Statistical Systems 2001 (NCSS, Kayville, UT) software [47]. Data were expressed as mean (SD). Comparisons between patients and healthy volunteers were done with the non-parametric Mann-Whitney U test.

#### Authors' contributions

MM, FIB, BH, DT, JM, LB, VR and TP elaborated the protocol and wrote the manuscript; FIB, DM, AP and BH performed the assays. TP and MM performed the statistical analysis. MJ elaborated the dietary questionnaire.

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