Circadian, Week-to-Week, and Physical Exercise-Induced Variation of Serum Microfibrillar-Associated Protein 4

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ABSTRACT: Serum microfibrillar-associated protein 4 (sMFAP4) has been investigated as a biomarker for various diseases and is demonstrated to show significant gradual increase with severity of liver fibrosis. Ideal biomarkers used for disease diagnosis or prognosis should display deviating levels in affected individuals only and be robust to factors unrelated to the disease. Here we show the impact of normal physiological variation of sMFAP4 by characterizing the circadian variation, week-to-week variation, and physical exercise-induced levels. Serum samples from 3 groups of healthy volunteers were drawn: 7 times during a 24-hour period, 5 times during a 3-week period, and before and after a standardized physical exercise challenge. sMFAP4 was determined by AlphaLISA. Statistical analysis was performed using mixed effects modeling of repeated measurements. Circadian variation of sMFAP4 was demonstrated, with time of peak and nadir values depending on age and gender. For males, the peak values were observed during nighttime whereas for females, peak values were observed in the morning. Individual sMFAP4 levels remained stable over a period of 3 weeks and physical exercise inferred a mild negative influence. In conclusion, the circadian sMFAP4 variation was significant, and the levels could be influenced by physical activity. However, these variations were of limited magnitude relative to previously observed disease-induced levels in support of the biomarker potential of sMFAP4.

KEYWORDS: MFAP4, biomarker, Serum, Constitutional level variation

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Introduction

Microfibrillar-associated protein 4 (MFAP4) is an extracellular matrix protein, which binds to extracellular matrix (ECM) fibers like collagen, elastin and fibrillin.[1-6] MFAP4 is localized primarily to sites rich in elastic fibers comprising aorta,[4,7,8] skin,[5,6,9] and a range of internal organs including lung, intestine, kidney, spleen, liver, and heart.[1,3,10] Immunohistochemical staining reveals a major site of expression in blood vessels, where MFAP4 is synthesized by vascular smooth muscle cells.[1] Furthermore, MFAP4 synthesis is demonstrated in dermal fibroblasts[5] and hepatic stellate cells.[11,12]

While embedded in the ECM, MFAP4 exposes a cell adhesion motif (RGD-sequence) enabling integrin ligation and focal adhesion for cellular activation.[13] MFAP4 is ligand for integrins $\alpha_V\beta_{3/5}$.[13,14] Mice with genetic ablation of MFAP4 are protected from aberrant healing responses when challenged by vascular injury,[13] chronic airway inflammation[14] or fibrosis.[15,16] In these pathologies, MFAP4-deficiency decreases inflammation, vascular and bronchial remodeling and fibrotic deposition.

Soluble MFAP4 has been detected in bronchoalveolar lavage[17] and in blood.[1] These levels of soluble MFAP4 are likely to partly represent turnover of ECM. However, the level of serum MFAP4 also varies with sex, age and smoking,[18] and is moderately affected by the presence of stable atherosclerosis,[1] peripheral artery disease,[19] presence of abdominal aorta aneurysms,[20] asthma,[21] and chronic obstructive lung disease[22] in addition to more markedly induced levels in liver fibrosis/cirrhosis.[11,23-26]

Considering sMFAP4 as a biomarker for chronic fibrosing disease warrants detailed evaluation of both technical and normal physiological variation. In this regard, we have previously shown that MFAP4 has a remarkable pre-analytical stability.[18] The aim of the present study was thus to determine the sMFAP4 variation in healthy persons both during a single day, throughout a period of 3 weeks, and before and after standardized physical activity.

Materials and Methods

Study populations

Peripheral blood samples from healthy persons in 3 different settings were collected as previously described.[27] The study was

carried out according to the Helsinki declaration; all participants gave their signed informed consent. The study protocol was approved by the Regional Ethics Committee (J. No. KF 01-138/03 and M02359-02).

Circadian variation of sMFAP4

Fifteen healthy individuals were admitted to Hvidovre hospital for successive blood sampling. Six males and 9 females with a median age at 50 years (range 32-66) were included. The subjects were self-reportedly healthy and did not take any medication besides non-prescription drugs. Blood samples were drawn 7 times during a 24-hour period, starting at 10 AM followed by samples every 3 hours until 10 pm. Time and numbers of meals were standardized, but meal size was individual. A fasting sample was drawn the following day at 7 AM before getting out of bed and was followed by a non-fasting sample at 10 AM. sMFAP4 measurements from 3 individuals at 10 AM the following day were not available. During the daytime, the participants were allowed to do normal activities, but they did not participate in heavy physical activities such as running.

Week-to-week variation of sMFAP4

Thirty-two healthy individuals (13 men and 19 women) with median age 42.5 years (range 24-66) were included. Eight persons had minor diseases with only rare and mild symptoms (seasonal allergy, mild arterial hypertension and intermittent minor reflux symptoms). Five blood samples were collected from each individual over 3 weeks. Samples were collected at 8 AM initially, 1 day later and subsequently after 1, 2, and 3 weeks.

Physical exercise-induced variation of sMFAP4

Thirteen healthy individuals (4 men and 9 women) with a median age of 50 years (range 35-64 years) were included and performed a biphasic exercise program using a bike-test (Monarch Viktergometer model 90814 E). The first part of the program was a 5-minute warming-up session, aiming to reach a submaximal level at 70%-80% of their maximal pulse capacity. The submaximal level was maintained for 20 minutes with 4 minutes cycles with increasing loads of 0.5, 0.7, 0.9, 1.1, and 1.3 kg, respectively. The participants started the exercise program in the period from 6.30 AM to 9.45 AM. Blood samples were collected prior to physical activity, immediately after, and subsequently 1 and 3 hours after cessation of the physical exercise.

Preanalytical handling and measurement of sMFAP4 by AlphaLISA

Peripheral blood samples were collected in endotoxin-free siliconized glass tubes without additives. The samples were kept at room temperature for 1 hour prior to centrifugation at 2,500 rpm for 10 minutes. Serum was collected and stored at −80°C until use. The AlphaLISA technique was based on inhouse produced mouse monoclonal antibodies HG-HYB 7-14 and HG-HYB 7-18 and used and validated for measuring of sMFAP4 as previously described.[1] HG-HYB 7–14 was conjugated to AlphaLISA Acceptor beads (Perkin Elmer) at a concentration of 0.1 mg antibody/mg acceptor beads following the manufacturer's instructions. HG-HYB 7–18 was modified by labeling with (+)-biotin N-hydroxysuccinimide ester (Sigma H1759) to permit binding to AlphaLISA streptavidin-coated donor beads. The AlphaLISA procedure was performed using 384-well microtiter plates (white opaque OptiPlateTM from Perkin Elmer) containing 5 ml of diluted serum (final dilution 1:100), 2 nM biotinylated HG-HYB 7-18, and 10 mg/ml HG-HYB 7-14 conjugated to Acceptor beads in a total of 20 ml AlphaLISAHHiBlock Buffer (PerkinElmer). The reaction mixture was incubated at room temperature for 60 minutes. Streptavidin donor beads were then added to reach a final concentration of 40 mg/ml, and the plate was incubated at room temperature in the dark for another 30 minutes, after which time it was read on an EnVision reader (PerkinElmer) using the AlphaScreen protocol. Briefly, the AlphaScreen protocol used AlphaScreen label 384-well Packard OptiPlates and the AlphaScreen 570 emission filter, a flash/time ratio of 0.55, a measurement height of 1 mm, an excitation time of 0.18 seconds, and an emission time of 0.37 seconds.

The experiments were performed in duplicate except for the standards and quality controls, which were performed in quadruplicate. The duplicate sample covariance was accepted if it was <10%. Standards were prepared by the serial dilution of MFAP4 overexpressing CHO cell culture supernatant in AlphaLISAHHiBlock Buffer. The concentration of the standard was estimated by the spiking of a known concentration of rMFAP4 into the samples. Standards included serial dilutions from 8000 to 7.8 mU/ml. The concentration of rMFAP4 was determined using Amino Acid Analysis by Alphalyse A/S (Odense, Denmark). When measured in serum, 1 U/ml = 38 ng/ml. Quality controls were diluted freshly for each run from frozen aliquots of a human serum pool and from frozen aliquots of rMFAP4 spiked pools. One control was prepared to contain a low content of human MFAP4 (QLow), and 2 controls were prepared using purified rMFAP4 (QMid) and (QHigh). The 3 quality controls were included in each plate. Interplate variation was accepted if all quality control measurements were within 2 standard deviations obtained from 10 consecutive runs. Within and between assay coefficient of variation was 8.7% and 9.5%, respectively.

Statistical methods

All statistical analyses were performed using STATA version 11.2 and R version 3.3.4 for Mac OS. The sMFAP4 measurements were normalized by a log-transformation. Mean levels and 95% confidence intervals (CI) were calculated at the log

Table 1. Circadian variation of sMFAP4.

TIME	SEX	EMPIRICAL			MODEL		
		MEAN	LOW	HIGH	MEAN	LOW	HIGH
10	F	12.91	10.35	15.31	11.56	9.73	13.73
13	F	11.00	9.00	13.64	11.12	9.33	13.25
16	F	10.11	7.07	13.34	10.06	8.48	11.94
19	F	9.72	7.80	12.67	9.07	7.66	10.75
22	F	9.60	7.01	12.47	8.67	7.24	10.38
7	F	10.38	8.87	12.42	11.04	9.28	13.13
10	F	14.92	11.05	19.72	11.56	9.73	13.73
10	M	9.27	8.36	10.24	9.83	8.25	11.72
13	M	9.21	8.24	10.27	9.29	7.76	11.11
16	M	7.29	6.40	8.75	9.17	7.71	10.89
19	M	8.68	7.17	11.20	9.52	8.04	11.28
22	M	10.08	8.39	11.92	10.19	8.48	12.24
7	M	8.61	6.79	11.18	10.52	8.84	12.52
10	M	10.22	8.46	13.26	9.83	8.25	11.72

Data is presented as geometric means of sMFAP4 (U/ml) with the lowest (Low) and highest (High) value of 95% confidence intervals. Empirical means and mixed effects regression model estimated means are shown.

scale and subsequently back-transformed to the scale of observations. The statistical questions addressed in this paper used a sampling structure with multiple measurements for each individual. Measurements from the same individual will be correlated, and data were consequently analyzed by mixed effects regression models. Sex and age were included as covariates. Time was included as a simple linear effect or in terms of trigonometric functions to accommodate periodic variations. For assessing the potential effect of physical activity upon the sMFAP4 level, we considered the persons to be in different states before and after the physical activity. The first measurement represented pre-physical activity and was drawn just before the activity commenced. Potential seasonal effects in the data on physical activity were accounted and tested for in our models. Intra-individual coefficient of variation (CV) was the mean logarithmically transformed sMFAP4 divided by the standard deviation for all measures performed on each subject. Confidence limits for the empirical means were obtained by bootstrapping within each time point with a bootstrap replication of 4000 samples.

All final models were tested for goodness of fit by residual plots.

Results

Circadian variation of sMFAP4

Mean sMFAP4 measurements obtained during a 24-hours period are summarized in Table 1.

Mixed effects linear regression using age, gender and time of the day as covariates, revealed a complex pattern for the circadian variation of the sMFAP4. Harmonic curves showed good fit. Time of peak and nadir and amplitude varied with age and gender (Figure 1A and D).

For a 50-year-old male the time of nadir and peak were estimated to 4 PM and 4 AM respectively, with sMFAP4 values of 9.4 U/ml and 10.4 U/ml respectively. For a 50-year-old female, the corresponding numbers were 10:20 PM and 10:20 AM with nadir and peak values of sMFAP4 of 8.9 U/ml and 11.6 U/ml respectively.

The magnitude of the circadian variation appeared to increase with age for females and decrease for males. In addition, the time points of nadir and peak increased with age, however most pronounced for males.

Week-to-week variation of sMFAP4

Mean sMFAP4 measurements for each sampling time during a 3-week period of observation are shown in Table 2. A daily increase in sMFAP4 of 4‰ was estimated using a mixed effects linear regression model. However, there was considerable variation, both within and between individuals, as can be seen in Figure 1B and E. No effects of age or gender could be detected. Mean sMFAP4 intra-individual coefficient of variation (CV) was determined to be 23.3% (SD ± 10.7%).

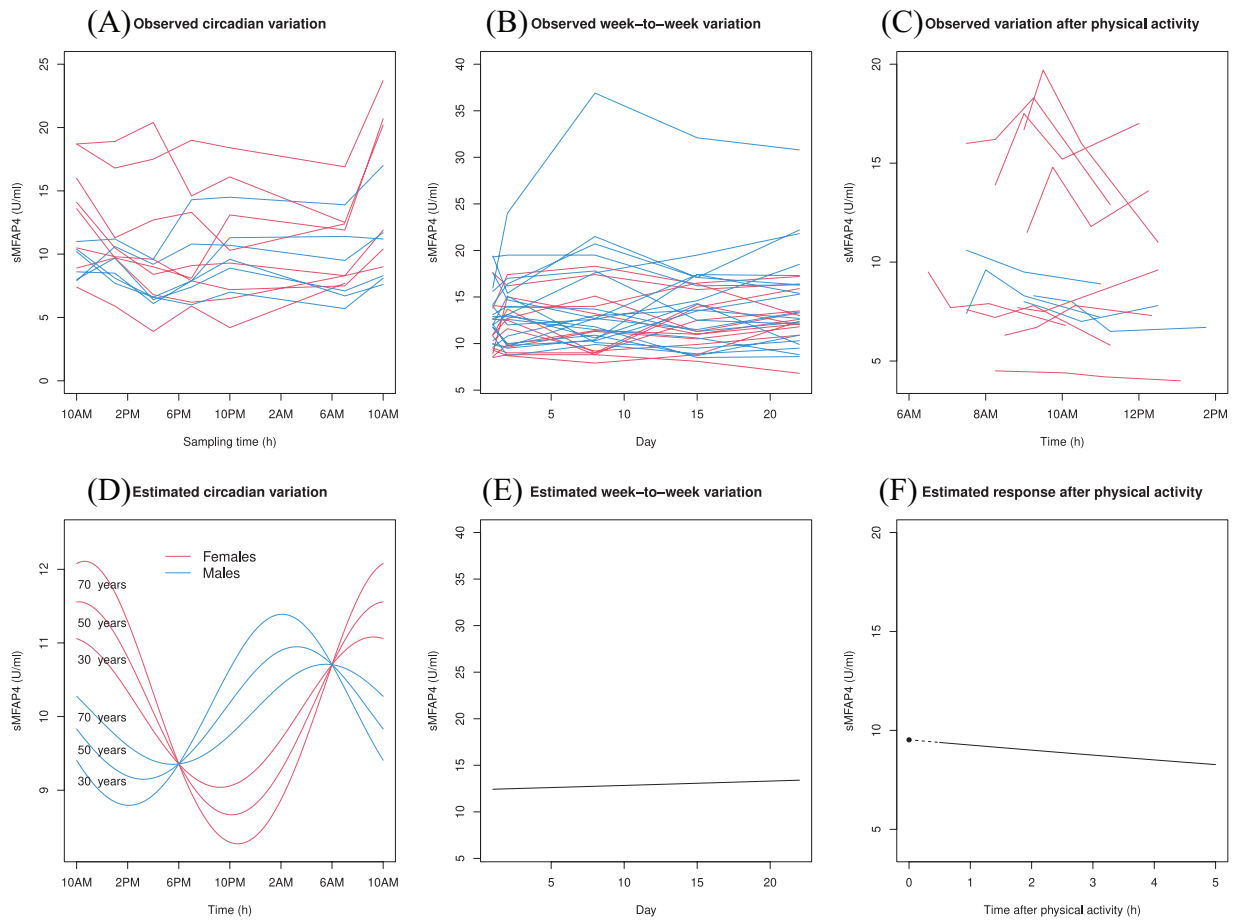

Figure 1. Variation of sMFAP4. Microfibrillar associated protein 4 (MFAP4) was measured in serum collected from 3 groups of volunteers to determine empirical variation of serum MFAP4 (sMFAP4) due to: (A) circadian variation, (B) week-to-week variation, and (C) variation after physical activity. Moreover, model estimated variation of sMFAP4 is shown for: (D) circadian variation, (E) week-to-week variation, and (F) variation after physical activity. Red = women, blue = men.
Abbreviations: h, hour; U/ml, units pr. ml.

Table 2. Week-to-week variation of sMFAP4.

DAY	EMPIRICAL			MODEL		
	MEAN	LOW	HIGH	MODEL	LOW	HIGH
1	6.41	5.21	7.78	12.43	11.35	13.62
2	8.55	7.37	9.62	12.47	11.4	13.65
8	8.22	7.44	8.99	12.75	11.68	13.91
15	8.06	7.05	9.21	13.07	11.96	14.28
22	8.79	7.60	10.13	13.4	12.18	14.75

Data is presented as geometric means of sMFAP4 (U/ml) with the lowest (Low) and highest (High) value of 95% confidence intervals. Empirical means and mixed effects regression model estimated means are shown.

Physical exercise induced variation of sMFAP4

Mean sMFAP4 measurements at each sampling time, before and after physical exercise, are shown in Table 3.

Individual and mean variations are shown in Figure 1C and F. An initial mixed effects linear regression included pre/post-state, effects of circadian variation, and time after physical activity. Only time after physical activity appeared to have a significant effect. The sMFAP4 level decreased by 2.8% per hour ($P = .03$) as compared to the initial measurement. The sera were collected at varying times of the day; however, the circadian pattern observed in the study above was not detected. Similarly, we found no effect of data being collected at varying seasons over the year. Still, these effects might add to the random variation between individuals, which constituted 94% of

Table 3. Physical exercise-induced variation of sMFAP4.

SAMPLE	EMPIRICAL			MODEL		
	MEAN	LOW	HIGH	MEAN	LOW	HIGH
Right before	9.23	7.64	11.28	9.26	8.45	10.14
Right after	9.49	7.62	12.01	9.00	8.22	9.85
1 h after	9.23	7.36	11.70	8.75	8.00	9.57
3 h after	8.50	6.97	10.49	8.51	7.78	9.30

Data is presented as geometric means of sMFAP4 (U/ml) with the lowest (Low) and highest (High) value of 95% confidence intervals. Empirical means and mixed effects regression model estimated means are shown.

the total random variation. The inter-individual variation was larger among females than males; this is in accordance with the observation in the study on circadian variation.

Model controls

The final model in each of the 3 sub-studies were checked for goodness of fit, by means of residual plots. In all cases we obtained good fits and this part of analysis indicated no cause for concern of the chosen models.

Discussion

In the present study of the normal physiological variation of sMFAP4, we demonstrate a slight increase in the marker during the observation period of 3 weeks, decrease with physical activity, and slight circadian variation. Moreover, we estimated a mean sMFAP4 intra-individual CV of 23.3% using measurements obtained at 8 AM during the three-week period.

The observed CV compares with our previous observation that plasma MFAP4 varied less than 25% between the baseline and a 3-month follow-up in 83% of patients that presented with stable chronic obstructive lung disease at enrolment.[22] All participants in the study regarding long-term variation were included at the same time of the year; therefore, no definite conclusions regarding seasonal variation can be reached. Peak values were found during nighttime/morning followed by a gradual decline during daytime. Time of peak and nadir differed between males and females and also, a larger magnitude of variation was seen among females. Moreover, variation increased with increasing age for females whereas variation decreased with age for males. The mechanisms underlying these different patterns of diurnal rhythmicity of sMFAP4 are unknown but may reflect parallel oscillations in hormonal or cytokine regulators. Moreover, it is not known whether the variation of sMFAP4 during the day is influenced by dietary intake. However, the larger inter-individual variation among females is in accordance with previous observations among twins, finding sMFAP4 level to be influenced by tobacco smoking in interaction with gender,[18] with tobacco smoking causing a larger decrease in sMFAP4 in females than males. In the present study, no information regarding tobacco smoking was available, thus, it can only be speculated whether smoking may account for the observed variation among females. Moderate physical activity induced a limited significant decrease in the hours following exercise. There was no detailed information about the sMFAP4 level from time zero (just before physical activity) and until the first post physical activity measurement. Hence, we could not make inference about how the sMFAP4 level evolved in the time span of activity.

Previously, we identified sMFAP4 as a biomarker candidate for liver fibrosis and cirrhosis in hepatitis C patients possibly reflecting the underlying ECM metabolism. sMFAP4 was able to reliably identify patients with severe fibrosis stages[11,25,26,28] in these studies. The accepted best available standard for determining the presence and degree of liver fibrosis is liver biopsy, despite well-documented limitations regarding sampling and interpretation variation as well as procedure related complications.[29,30] These limitations have encouraged the development of reliable biochemical markers to reduce the need to perform liver biopsies, and to cover the need for biomarkers to diagnose fibrosis, to stage the fibrosis, and to grade current fibrogenesis. Despite the numerous biochemical biomarkers and panels reported none has yet proven ideal.[31] One of the obstacles is the analytical imprecision, which was recently described to be relatively high for example, for the biomarker hyaluronic acid in healthy subjects as well as patients suffering from hepatitis.[32] Thus, a CV of 62% for hyaluronic acid was reported for healthy subjects and 38% for patients suffering from hepatitis C.[32] Compared to these observations, the CV for sMFAP4 presented here along with the pre-analytical and biological stability and a significant, yet, limited circadian variation support a proposed biomarker potential of sMFAP4.

sMFAP4 is robust to preanalytical sample handling.[18] Thus, in clinical practice the mild changes (few units) in sMFAP4 due to physical activity or over intervals of weeks may be negligible compared to the log-fold increased levels found in fibrotic liver disease.[25] In contrast, the diurnal variation suggests all blood sampling is performed at the same time of day.

Limitations of the study include the lack of detailed information on tobacco smoking history and the limited numbers of subjects. Moreover, samples from the 3 different populations were stored frozen for different periods and this may have

contributed to the different mean sMFAP4 levels observed in the 3 populations. However, it should be underscored that samples from each sub-study were analyzed using reagents from the same batches.

In summary, sMFAP4 remains relatively stable at the individual level over a prolonged period of time yet exhibiting circadian variation. The latter variation of sMFAP4 emphasizes the importance of standardized blood sampling conditions in future studies on sMFAP4 as biomarker of fibrosis.

Data access

Contact corresponding author for data access.

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