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ORIGINAL ARTICLE

Circadian time structure of fatty acids and vascular monitoring

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Summary
The circadian variation of 40 circulating fatty acids related variables was assessed from one man (F) and one woman (G). Each provided blood samples by finger pricking at about 4-hour intervals for 24 hours. A statistically significant rhythm was found in 65% of the variables after data expressed as a percentage of their 24-hour mean values were pooled. In particular, a putative circadian rhythm for *n*-3 and *n*-6 fatty acids deserves exploration. The predominant 12-hour component found to characterize the *n*-3 status of G may stem from the odd schedule she followed on the day of study, as attested by alterations in the time structure of her blood pressure on the day of study, as compared to similarly collected data on 33 other Sundays in 2009 available as control information. Circadian vascular characteristics are sensitive markers of loads, including the rest-activity schedule.

Key words: blood pressure; cholesterol; circadian; highly unsaturated fatty acids (HUFA); omega-3 (*n*-3) and omega-6 (*n*-6) fatty acids

This paper, the fruit of international cooperation, is dedicated to the memory of Helena Rašková, the grande dame of international immunology, and concomitantly the prominent ambassador of Czech science abroad. In a dignified, unobtrusive way, her Prague home became a meeting place for local and international scientists, and the many connections she established continue fruitfully, but with an obvious great void.

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INTRODUCTION

Focus upon diet and circulating lipids has shifted from the assumption that a high fat diet raises blood cholesterol, which in turn is associated with conditions such as coronary heart disease, to include the putative role of *n*-3 (also referred to as omega-3 or ω-3) fatty acids in the modulation of cardiovascular functions (Dubnov et al. 2008, De Meester 2009, Simopoulos 2009). In the absence of data on dynamics, concepts such as "balance" and "homeostasis" are invoked (De Meester 2009, Simopoulos 2009), in good company with the aging Claude Bernard (1885), yet at variance with this eminent scientist's answer when asked earlier by the Journal d'Anatomie et de Physiologie what his major discoveries were. The younger Claude Bernard singled out "la variabilité immense du milieu intérieur" (Bernard 1865).

Our goal herein is to assess this variability, remembering Charles Chossat, who demonstrated that the circadian rhythm in cloacal temperature of pigeons deprived of all food and water persisted until the day of death from starvation and dehydration (Chossat 1843) (Fig. 1). Similarly, the circadian rhythm in liver glycogen was shown to persist during starvation (Ågren et al. 1931, Haus and Halberg 1966), indicating that feeding alone does not account for circadian rhythmicity in these variables (Higgins et al. 1932, 1933). Brillat-Savarin (1826) suggested that we are what we eat, but we must remember that we are also "when we eat" (Halberg et al. 1995). In an experimental model without big fat reserves, the timing of the availability of food could account for the difference between death and survival (Nelson et al. 1973).

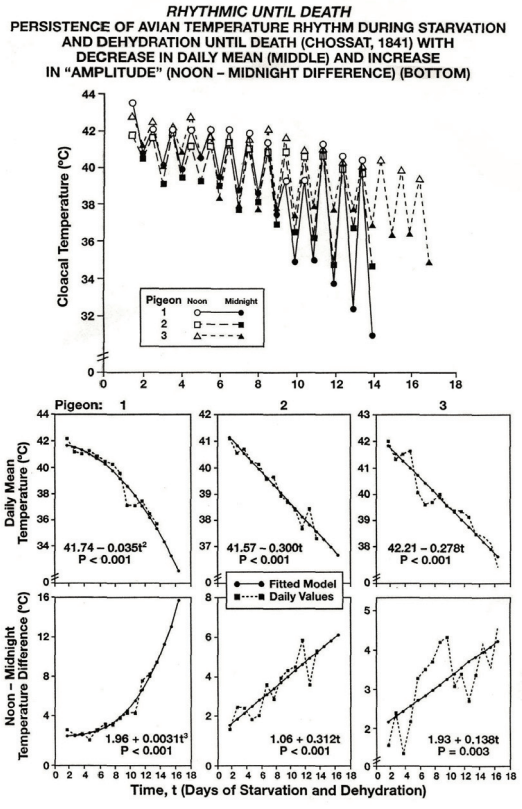

Fig. 1. **Demonstration by Charles Chossat** (1843) that the circadian rhythm (gauged by measurements at noon and midnight) of cloacal temperature of pigeons deprived of all food and water persists until the day of death from starvation and dehydration. Top: Records from 3 pigeons. Bottom: Changes in daily averages (row 1) and noon-midnight differences (row 2) as a function of time. Second-order polynomials fitted to these data indicate that while the average temperature decreases, the prominence of the circadian variation increases.

Variability, notably along the 24-hour scale has led to the formulation of circadian systems (Halberg 1959) and the birth of chronobiology (Halberg 1969), a discipline that eventually led to chronomics (Halberg et al. 2009) (Fig. 2). We need to lift the curtain of ignorance drawn over the range of physiologic variation (Fig. 3), to estimate in statistical inferential terms the predictable changes that can be anticipated to recur with time, and to replace concepts such as "balance" and "homeostasis" with maps of lawful time structures, the essence of chronobiology and chronomics, that lead to chronobioethics (Fig. 2). Computer-implemented hypothesis testing and parameter estimation thus becomes available to everyone for risk assessment and detection by affordable home-based self-surveillance, without the need to involve a care provider as long as abnormalities are not found (Sánchez de la Peña 2008, Halberg et al. 2009).

MATERIALS AND METHODS

On 27 Sep 2009, one man and one woman provided blood samples by finger pricking around the clock at approximately 4-hour intervals for 24 hours (6 samples each). Samples were collected at 01:30, 05:30, 10:00, 13:45, 17:45, and 21:30, before meals. Sleep was between 03:30 and 09:30, with one interruption at 05:30 for finger pricking and another around 07:30 for storing the samples after letting them dry at room temperature for about 2 hours. The man (F) is 61 years of age and has a history of insulin-dependent diabetes mellitus first diagnosed at about 21 years of age. He also takes daily doses of aspirin (81 mg in the morning) and Lovastatin (20 mg in the evening), preventively. The woman (G) is 59 years of age and is mostly clinically healthy but takes synthroid (0.175 mg/day) to treat hypothyroidism. She also takes calcium and vitamin D supplementation (Oysco 500/D 3 times a day) and Alendronate (35 mg/week), preventively. Blood samples from fingertips were adsorbed on a collecting kit (Sigma-Aldrich) and analyzed by gas chromatography for a direct evaluation of fatty acids (Marangoni et al. 2004, 2007, Risé et al. 2005, Yehuda et al. 2005, Lagarde 2008, Stark 2008, Galli and Calder 2009, Galli et al. 2009, Ratnayake and Galli 2009). Fig. 4 illustrates the metabolic pathways of poly-unsaturated fatty acids (PUFA) of the two series.

Blood glucose was also determined around the clock by F with a OneTouch UltraLink (Medtronic) glucometer. Systolic (S) and diastolic (D) blood

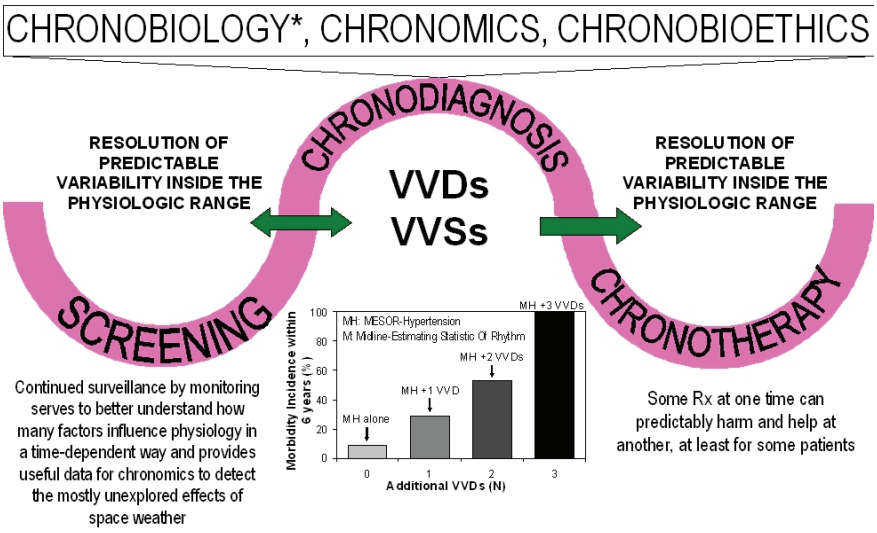

Fig. 2. **Scheme illustrating how the study of circadian systems and broader time structures led to the development of chronobiology, chronomics, and eventually to chronobioethics.** From a practical viewpoint, screening and continued surveillance is not restricted to single or mean values but to variability assessed in the light of time-varying reference values. Accordingly, the chronodiagnosis includes alterations in rhythm characteristics and other endpoints serving to guide the scheduling of any needed treatment (chronotherapy). In the case of blood pressure, screening for vascular variability disorders (VVDs) is important when outcome studies show that an elevated blood pressure not complicated by other VVDs is associated with a relatively small increase in cardiovascular disease risk. By contrast, when it is complicated by 1, 2 or 3 additional VVDs, the risk increases dramatically (box). Modified from De Meester (personal communication).
*Occasionally, human physiological vascular variability (VV) is transiently altered. Lasting alterations become a vascular variability disorder (VVD), or if VVDs coexist, a VV syndrome (VVS). VVDs and VVSs require detection and chronotherapy for prehabilitation (thereby reducing the need for rehabilitation; Halberg et al. 2008c, 2009).

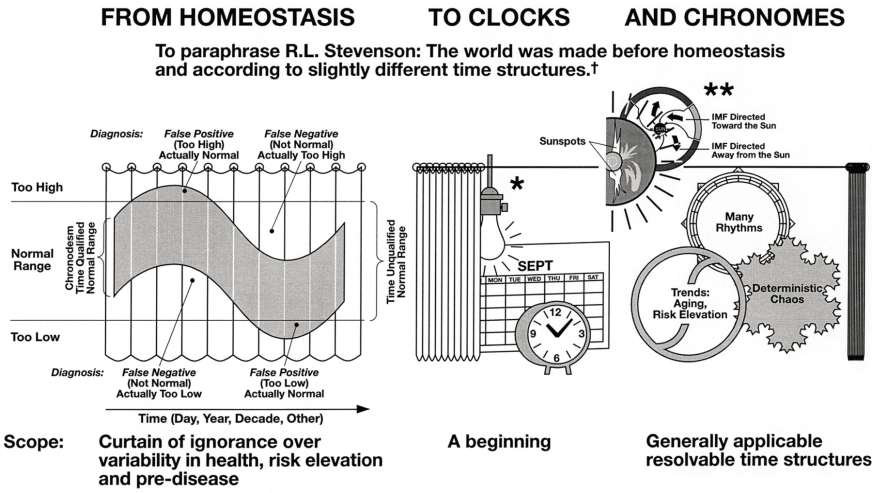

Fig. 3. **Abstract graph conveying the need to lift the curtain of ignorance drawn over the physiological range** within which much of the variability occurs in a predictable manner (left). Assessing the circadian and circannual variations (middle) is a welcome start that is best complemented by a rigorous assessment of much broader time structures that include the investigation of non-photic as well as photic solar influences on physiology and pathology (right).
*The "Master Switch", **Several switches, including helio-geomagnetics, † Inferential statistical methods map chronomes as molecular biology maps genomes; biologic chronomes await resolution of their interactions in us and around us, e.g., with magnetic storms in the interplanetary magnetic field (IMF).

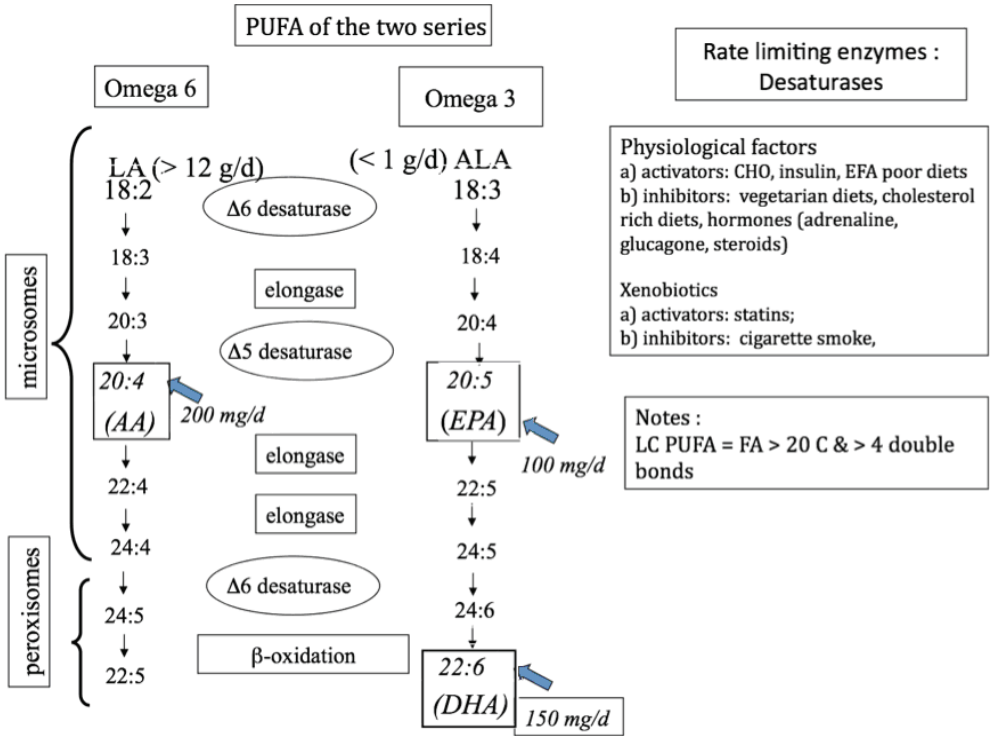

Fig. 4. **Illustration of the metabolic pathways of poly-unsaturated fatty acids (PUFA) assessed in around-the-clock samples from two subjects.** From Galli (personal communication).

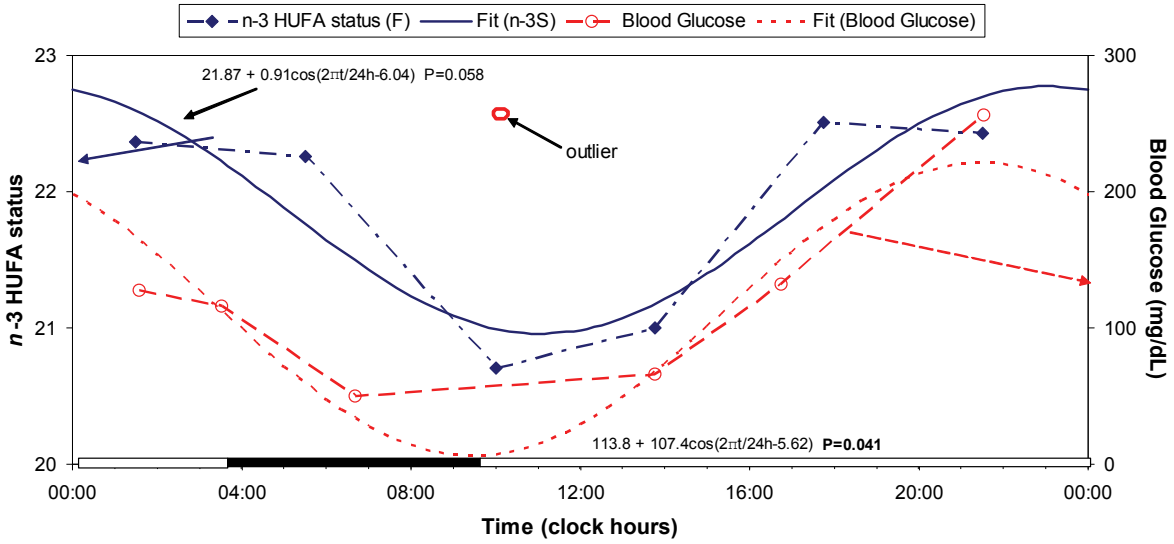

Fig. 5. **The circadian variation in *n*-3 HUFA status of F is similar to that of blood glucose**.

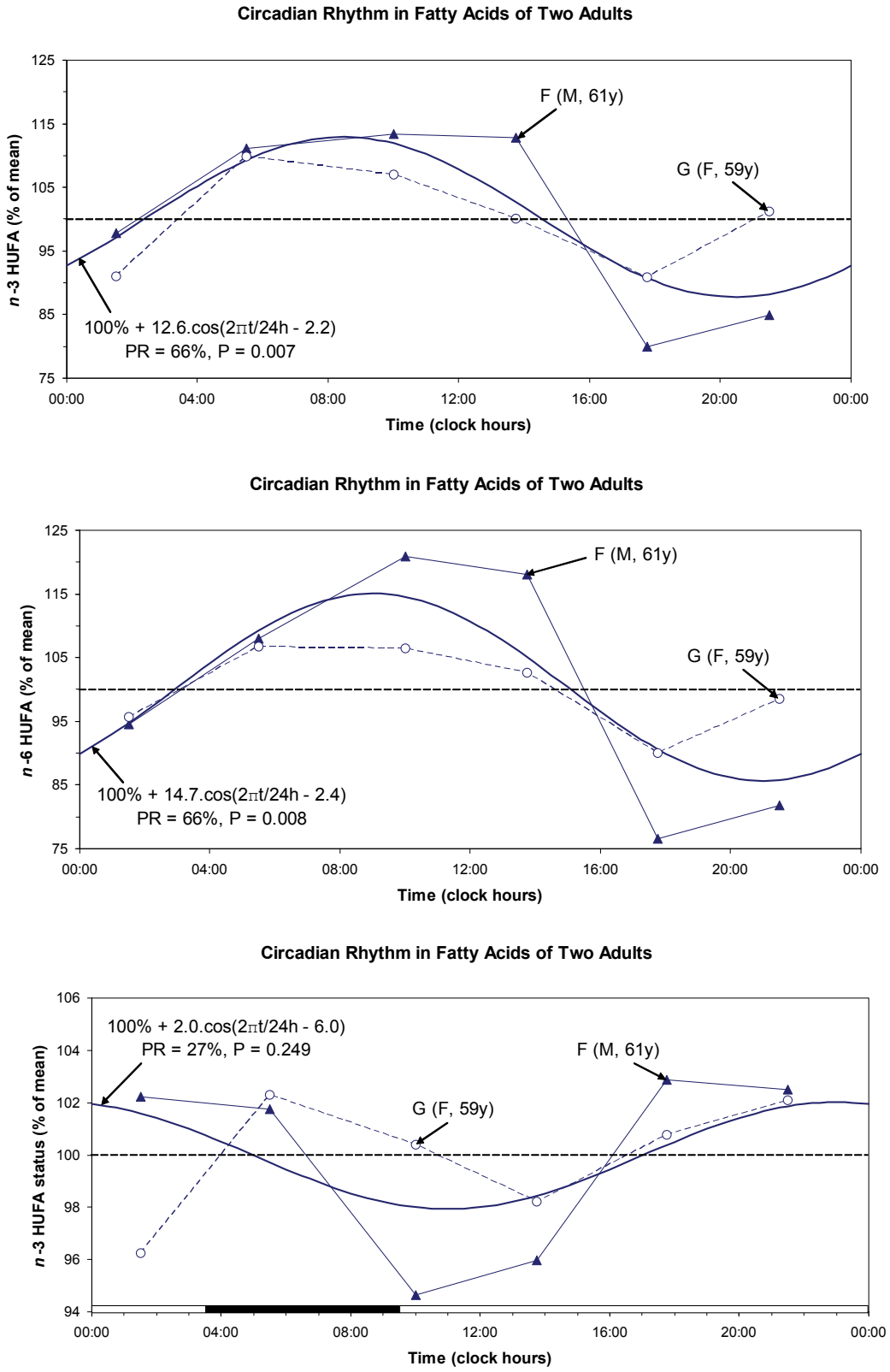

Fig. 6. **A circadian rhythm is detected for *n*-3 HUFA and *n*-6 HUFA** but not for *n*-3 HUFA status after pooling data of F and G expressed as a percentage of their respective mean value.

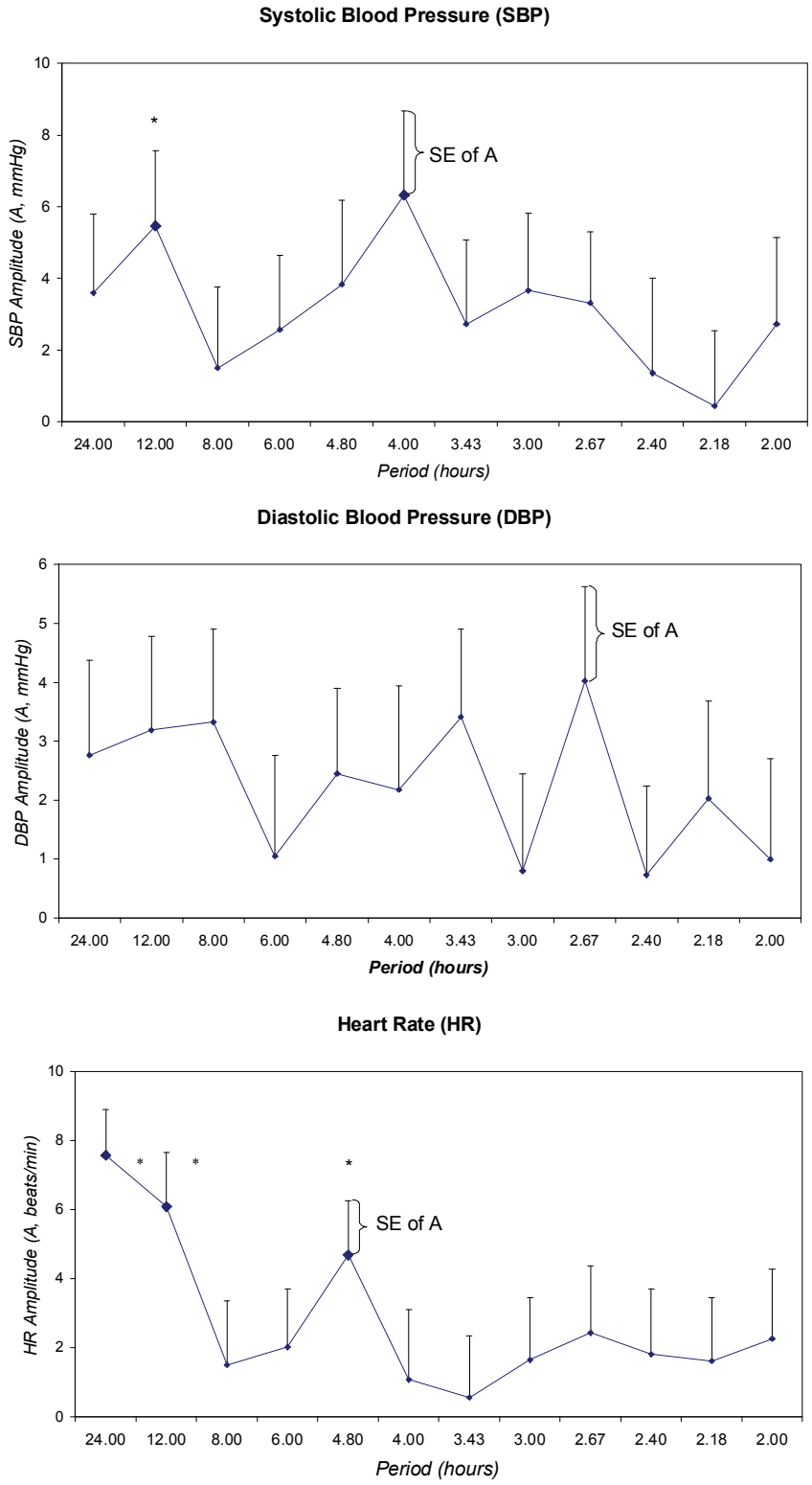

Fig. 7. **Least squares spectra of systolic (S) and diastolic (D) blood pressure (BP) and heart rate (HR) of G during the day of study.** A circadian rhythm is only detected for HR. In the case of SBP, the 12-hour but not the 24-hour component is statistically significant (*).

Table 1. **Individual cosinor results of fatty acid related variables** – summary at trial period of 24 hours*

	F (M, 61y)						G (F, 59y)					
Fatty acid	P	PR	M	A	Φ	Best fit	P	PR	M	A	Φ	Best fit
16:0	0.332	52	27.32	0.79	–316	12	0.043	88	25.96	0.64	–320	24
18:0	0.188	67	11.29	0.36	–259	24	0.366	49	11.97	0.15	–215	12
20:0	0.045	87	0.41	0.09	–117	24	0.393	46	0.45	0.04	–138	12
22:0	0.057	85	1.32	0.33	–124	24	0.110	77	1.69	0.30	–137	24
24:0	0.038	89	2.23	0.58	–135	24	0.071	83	2.02	0.43	–134	24
16:1	0.630	27	1.80	0.30	–70	12	0.718	20	1.79	0.06	–58	12
18:1	0.034	89	20.29	3.60	–311	24	0.127	75	19.27	1.31	–303	24
18:1 *n*-7	0.060	85	1.54	0.28	–278	24	0.610	28	1.67	0.12	–301	12
20:1	0.267	59	0.18	0.04	–179	24	0.673	23	0.15	0.02	–146	12
22:1	0.403	45	0.07	0.03	–137	12	0.017	93	0.07	0.03	–135	24
24:1	0.052	86	1.99	0.54	–135	24	0.095	79	2.25	0.42	–141	24
20:3 *n*-9	0.477	39	0.05	0.04	–209	12	0.076	82	0.05	0.02	–182	24
18:2 *n*-6	0.304	55	20.94	1.08	–112	24	0.845	11	19.59	0.21	–326	12
18:3 *n*-6	0.832	12	0.16	0.02	–205	12	0.279	57	0.30	0.10	–94	24
20:3 *n*-6	0.107	77	0.99	0.17	–144	24	0.363	49	0.93	0.05	–135	24
20:4 *n*-6	0.049	87	5.64	1.21	–138	24	0.165	70	7.36	0.48	–118	24
22:4 *n*-6	0.058	85	0.97	0.26	–131	24	0.292	56	1.12	0.15	–128	24
22:5 *n*-6	0.088	80	0.18	0.11	–164	24	0.519	35	0.16	0.03	–142	12
18:3 *n*-3	0.466	40	0.46	0.07	–28	12	0.407	45	0.38	0.11	–39	12
20:5 *n*-3	0.907	6	0.29	0.01	–250	12	0.857	10	0.50	0.01	–327	12
22:5 *n*-3	0.078	82	0.68	0.15	–129	24	0.349	50	0.93	0.10	–135	24
22:6 *n*-3	0.020	93	1.20	0.24	–127	24	0.334	52	1.40	0.12	–115	24
SFA	0.894	7	42.58	0.19	–226	12	0.788	15	42.09	0.20	–168	12
MUFA	0.070	83	25.87	3.10	–312	24	0.213	64	25.20	0.95	–297	24
PUFA	0.135	74	31.42	3.11	–127	24	0.285	57	32.41	0.74	–108	24
U.I.	0.106	78	112.1	7.90	–132	24	0.158	71	119.6	2.93	–112	24
n-6	0.131	74	28.88	2.74	–129	24	0.368	49	29.46	0.60	–109	24
n-3	0.118	76	2.63	0.38	–119	24	0.334	52	3.20	0.24	–97	24
n-6/*n*-3	0.155	71	11.05	0.63	–281	24	0.572	31	9.26	0.47	–274	12
DHA/AA	0.440	42	0.21	0.01	–26	24	0.759	17	0.19	0.003	–141	12
EPA/AA	0.077	82	0.05	0.01	–306	24	0.402	46	0.07	0.01	–322	12
n-3 HUFA	0.051	86	2.17	0.39	–129	24	0.334	52	2.83	0.21	–121	24
n-6 HUFA	0.053	86	7.78	1.74	–139	24	0.145	72	9.58	0.69	–122	24
n-3 HUFA status	0.107	77	21.87	0.91	–346	24	0.997	0	22.77	0.03	–211	12
EPA/ALA	0.474	39	0.63	0.08	–215	24	0.608	28	1.48	0.38	–192	12
AA/LA	0.052	86	0.27	0.05	–141	24	0.236	62	0.37	0.03	–118	24
AA/DHGLA	0.020	93	5.67	0.29	–120	24	0.413	45	7.93	0.22	–69	12
DHA/ALA	0.134	74	2.68	0.75	–154	24	0.370	48	4.10	1.28	–180	12
ALA/LA	0.466	40	0.02	0.003	–25	24	0.343	51	0.02	0.01	–26	24
DHGLA/LA	0.037	89	0.05	0.01	–144	24	0.637	26	0.05	0.003	–146	24

Terminology of fatty acids: The first number indicates the number of carbon atoms, the second number after ":" indicates the number of double bonds.

n-3 or *n*-6 indicate that the double bond closest to the methyl end (and most distant from the carboxyl end) of the molecule is 3 or 6 carbons away from the methyl end in the carbon chain of the fatty acid.

SFA: saturated fatty acids; MUFA: mono-unsaturated fatty acids; PUFA: Poly-unsaturated fatty acids; U.I.: unsaturation index [U.I. = (Sum of % of each fatty acid × its number of double bonds)/100)]; DHA: docosahexaenoic acid (22:6*n*-3); AA: arachidonic acid (20:4*n*-6); EPA: eicosapentaenoic acid (20:5*n*-3); HUFA: highly unsaturated fatty acids; ALA: α-linolenic acid (18:3*n*-3); LA: linoleic acid (18:2*n*-6); DHGLA: dihomo-gamma-linolenic acid (20:3*n*-6).

* P: P-value from zero-amplitude test; PR: percentage rhythm, proportion of variance accounted for by least squares fit of 24-hour cosine curve; M: MESOR, rhythm-adjusted mean; A: 24-hour amplitude, half the extent of predictable variation within a day; Φ: 24-hour acrophase, a measure of the timing of overall high values recurring each day; Best fit: period of 24-hour or 12-hour component accounting for the largest proportion of overall variance.
Results for UI, DHA/AA, EPA/AA, EPA/ALA, AA/LA, AA/DHGLA, ALA/LA, and DHGLA/LA, referring to selected ratios between PUFA relevant as indices of metabolic steps (product/precursor ratios) or as ratios between relevant PUFA of the *n*-3 and *n*-6 series (e.g., EPA, DHA and AA), are included for completeness, even though they are not directly pertinent to the major topic of this paper.

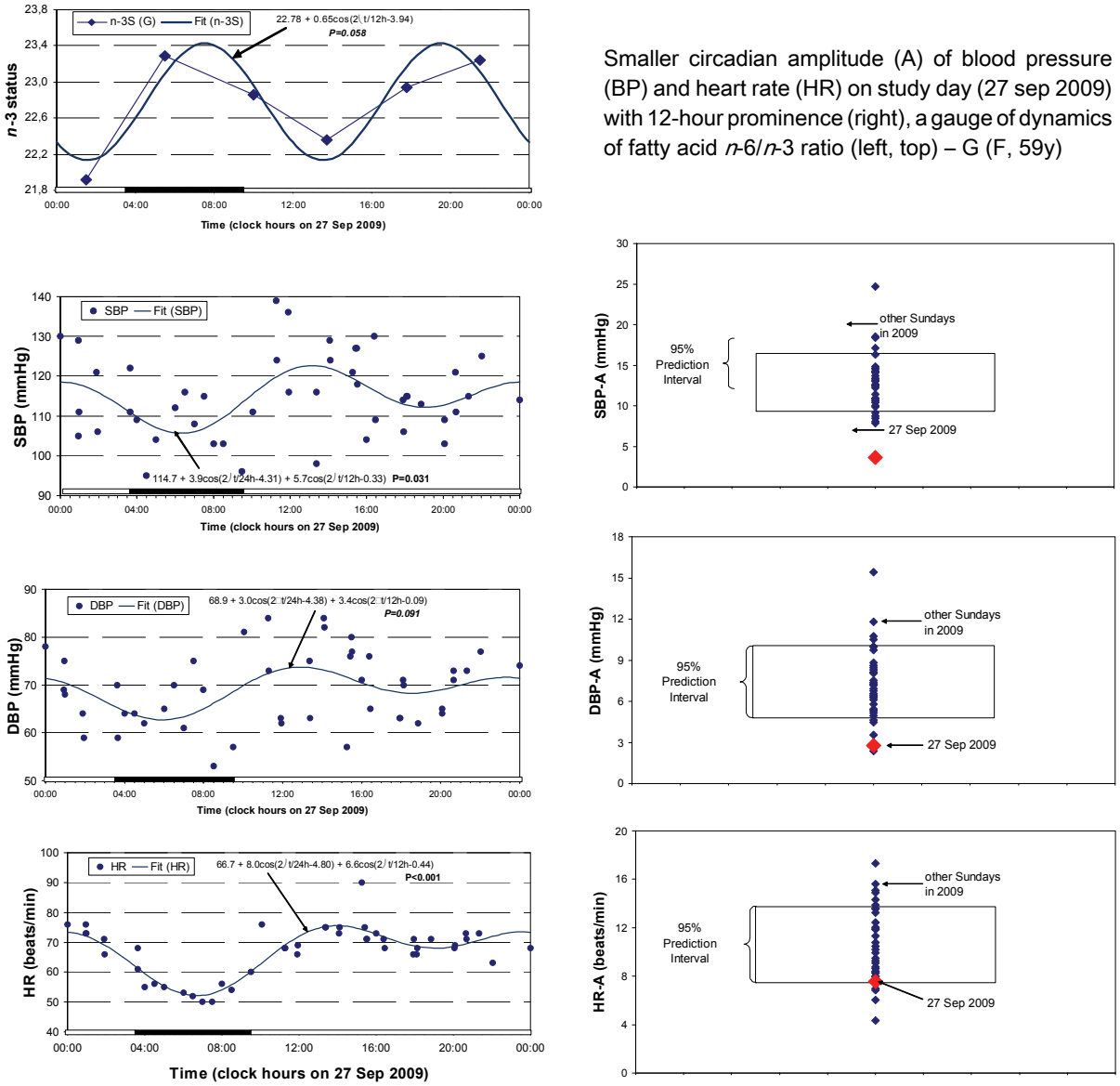

Fig. 8. **The odd schedule of G on the day of study may account for the 12-hour prominence in her records of *n*-3 HUFA status and systolic (S) blood pressure (BP).** By comparison to the distribution of 24-hour amplitudes of SBP, diastolic (D) BP and heart rate (HR) on all other Sundays in 2009, those on the day of study were lowest for BP and among the lowest for HR, lying below the lower 95% prediction limit of BP amplitudes.

Table 2. **Cosinor results of fatty acid related variables of pooled data expressed as a percentage of their respective 24-hour mean values** – summary at trial period of 24 hours*

Fatty acid	P	PR	A	Φ	Best fit
16:0	0.013	62	2.66	–318	24
18:0	0.054	48	2.10	–247	24
20:0	0.010	64	14.14	–124	24
22:0	<0.001	79	21.48	–129	24
24:0	<0.001	85	23.81	–135	24
16:1	0.411	18	10.00	–68	12
18:1	0.003	72	12.19	–309	24
18:1 *n*-7	0.027	55	12.50	–285	24
20:1	0.151	34	17.21	–167	24
22:1	0.012	63	43.52	–137	24
24:1	<0.001	81	22.98	–137	24
20:3 *n*-9	0.215	29	51.89	–198	24
18:2 *n*-6	0.469	15	2.15	–103	12
18:3 *n*-6	0.475	15	15.86	–114	12
20:3 *n*-6	0.022	57	11.01	–142	24
20:4 *n*-6	0.010	64	13.87	–133	24
22:4 *n*-6	0.005	69	19.95	–130	24
22:5 *n*-6	0.031	54	39.12	–159	24
18:3 *n*-3	0.105	39	21.57	–34	24
20:5 *n*-3	0.803	5	2.30	–300	24
22:5 *n*-3	0.008	66	16.75	–131	24
22:6 *n*-3	0.004	70	14.23	–124	24
SFA	0.703	8	0.40	–196	12
MUFA	0.012	63	7.82	–308	24
PUFA	0.039	51	6.05	–123	24
U.I.	0.015	61	4.70	–127	24
n-6	0.044	50	5.73	–125	24
n-3	0.015	61	10.79	–111	24
n-6/*n*-3	0.068	45	5.34	–278	24
DHA/AA	0.501	14	1.99	–48	12
EPA/AA	0.008	65	16.22	–308	24
n-3 HUFA	0.007	66	12.59	–127	24
n-6 HUFA	0.008	66	14.70	–135	24
n-3 HUFA status	0.249	27	2.04	–345	12
EPA/ALA	0.250	27	19.17	–200	12
AA/LA	0.004	71	12.20	–137	24
AA/DHGLA	0.023	57	3.57	–102	24
DHA/ALA	0.030	54	28.89	–168	24
ALA/LA	0.132	36	20.61	–30	12
DHGLA/LA	0.016	60	9.43	–148	24

For terminology of fatty acids, see Table 1.

* P: P-value from zero-amplitude test; PR: percentage rhythm, proportion of variance accounted for by least squares fit of 24-hour cosine curve; M: MESOR, rhythm-adjusted mean; A: 24-hour amplitude, half the extent of predictable variation within a day; Φ: 24-hour acrophase, a measure of the timing of overall high values recurring each day; Best fit: period of 24-hour or 12-hour component accounting for the largest proportion of overall variance.

Results for UI, DHA/AA, EPA/AA, EPA/ALA, AA/LA, AA/DHGLA, ALA/LA, and DHGLA/LA, referring to selected ratios between PUFA relevant as indices of metabolic steps (product/precursor ratios) or as ratios between relevant PUFA of the *n*-3 and *n*-6 series (e.g., EPA, DHA and AA), are included for completeness, even though they are not directly pertinent to the major topic of this paper.

40 variables (80%). On the average, the circadian variation accounts for a predictable excursion of 14.3% around the 24-hour mean value (standard deviation: 11.4%), and up to 51.9%.

Table 3. **Average fatty acid (FA) composition (% of total FA) in whole blood drops in a reference Italian population.**

Fatty acid	Men (N = 322)		Women (N = 328)		
	Mean	SE	Mean	SE	
16:0	22.93	0.12	22.52	0.11	
18:0	11.74	0.09	11.66	0.06	
20:0	0.42	0.00	0.45	0.00	
22:0	1.28	0.02	1.33	0.01	
24:0	2.56	0.04	2.42	0.03	
16:1	1.22	0.03	1.21	0.03	
18:1	21.61	0.17	20.77	0.15	**
18:1 *n*-7	1.74	0.02	1.71	0.02	
20:1	0.20	0.00	0.19	0.00	
22:1	0.50	0.02	0.52	0.02	
24:1	3.18	0.05	3.13	0.04	
20:3 *n*-9	0.20	0.02	0.19	0.01	
18:2 *n*-6	16.78	0.17	18.50	0.16	**
20:3 *n*-6	1.45	0.02	1.50	0.02	*
20:4 *n*-6	8.53	0.08	8.44	0.07	
22:4 *n*-6	1.24	0.03	1.13	0.02	
22:5 *n*-6	0.24	0.01	0.22	0.01	
18:3 *n*-3	0.29	0.01	0.31	0.01	
20:5 *n*-3	0.44	0.02	0.46	0.02	
22:5 *n*-3	0.92	0.02	0.91	0.02	
22:6 *n*-3	1.95	0.04	1.88	0.04	
SFA	39.26	0.14	39.24	0.16	
MUFA	27.99	0.18	27.79	0.18	
PUFA	32.75	0.19	32.96	0.20	
n-6	28.98	0.18	29.21	0.20	
n-3	3.58	0.06	3.56	0.06	
n-6/*n*-3	8.09	0.00	8.20	0.00	
n-3 HUFA	11.46	0.11	11.30	0.09	
n-6 HUFA	3.31	0.06	3.26	0.06	
n-3 HUFA status	22.34	0.34	22.15	0.31	

For terminology of fatty acids, see Table 1.
* P<0.01; ** P<0.001 from comparison of values for F and G with results from reference Italian population (Galli et al. 2009). Results for UI, DHA/AA, EPA/AA, EPA/ALA, AA/LA, AA/DHGLA, ALA/LA, and DHGLA/LA, referring to selected ratios between PUFA relevant as indices of metabolic steps (product/precursor ratios) or as ratios between relevant PUFA of the *n*-3 and *n*-6 series (e.g., EPA, DHA and AA), are included for completeness, even though they are not directly pertinent to the major topic of this paper.

pressure (BP) and heart rate (HR) were automatically measured around the clock at about 30-min intervals by G with an ambulatory monitor (TM-2430) from A&D (Tokyo, Japan).

Each data series was analyzed by the extended cosinor (Halberg 1980, Cornélissen and Halberg 2005, Refinetti et al. 2007). A 24-hour (or 12-hour) cosine curve was fitted by least squares to each variable, yielding estimates of the MESOR (Midline Estimating Statistic Of Rhythm, M; usually more precise and more accurate than the arithmetic mean), the double circadian amplitude (2A, an estimate of the predictable extent of change within a cycle), and the circadian acrophase (Φ, an estimate of the timing of overall high values recurring in each cycle).

In view of the relatively small number of determinations for each individual time series, the data from F and G were pooled after being expressed as a percentage of their respective mean value. Cosinor analyses were repeated on these pooled relative data with trial periods of 24 and 12 hours. Testing was at the significance level $2\alpha = 0.05$. The

n-3 and *n*-6 HUFA (Highly Unsaturated Fatty Acids) and their (*n*-6/*n*-3) ratio (*n*-3 status) were also fitted with a composite model consisting of cosine curves with periods of 24 and 12 hours.

RESULTS

The circadian rhythm characteristics of all 40 fatty acid related variables assessed by gas chromatography are listed for both F and G in Table 1. A circadian rhythm could be demonstrated with statistical significance for 7 variables for F and for 2 variables for G. In only 8 of 40 variables was the 12-hour component more prominent than the 24-hour one for F, whereas for G, the 24-hour component was the most prominent one in only 23 of the 40 variables, Table 1.

As seen from Fig. 5, in the case of F, the circadian variation in the *n*-3 HUFA status is similar to that of blood glucose, also measured by finger prick, after removal of an outlier resulting from an over-compensation of a hypoglycemic episode. The circadian variation in blood glucose is detected with statistical significance.

After expressing the data as a percentage of their respective mean values and pooling the data from F and G for each variable, a circadian rhythm is detected in 26 cases (65%, Table 2). Statistical significance is thus reached in many more cases than the 5% expected by chance alone. Moreover, the 24-hour component predominates in 32 of the 40 variables (80%). On the average, the circadian variation accounts for a predictable excursion of 14.3% around the 24-hour mean value (standard deviation: 11.4%), and up to 51.9%.

The individual data and the 24-hour cosine curve fitted to the pooled relative values of *n*-3 HUFA, *n*-6 HUFA, and *n*-3 HUFA status are illustrated in Fig. 6. A circadian rhythm is readily apparent for both *n*-3 HUFA and *n*-6 HUFA. This component was already detected with borderline statistical significance for F. Statistical significance is not reached, however, for their ratio (*n*-3 status). One reason may stem from the fact that n-6 and *n*-3 change in the same direction. Synchronized changes of *n*-6 and *n*-3 may be due to changes of lipoproteins that contain both of them in a given ratio. Another reason may stem from the lack of statistical power related to the small number of samples. In the case of F, lower values are found around mid-day as compared to evening and night. The disturbed schedule on the day of sampling, notably in the case of G, may also have played a role, as the 12-hour component accounted for a larger proportion of the overall variability than the 24-hour component in her case.

DISCUSSION

The presence of spontaneous periodic changes in a number of variables, not only occurring in the absence of loads ("stress"), but sometimes seemingly increasing in (or at least being associated with) a very prominent amplitude when the load of repeated sampling was removed, was reported over half a century ago (Halberg and Visscher 1950, see also Halberg et al. 2003), an important observation as such, noted by Aschoff (1954). The demonstration that these rhythms are sensitive gauges of loads consisted of the finding that the handling of a mouse for a single venisection (and blood sampling for the counting of eosinophil cells) carried out at intervals of several days sufficed to result in eosinopenia (Halberg 1953).

An odd schedule for G on the day of blood sampling may account, at least in part, for a 12-hour over 24-hour predominance in her *n*-3 index, a marker of the *n*-3 HUFA status, and in her BP but not in her HR data. Whereas HR was characterized by a statistically significant circadian variation, the 24-hour component was not found for BP, as illustrated in the least squares spectra of Fig. 7. The least squares fit of a two-component model consisting of cosine curves with periods of 24 and 12 hours accounts for 75% of the overall variance in the case of HR, but only for 22% and 17% for SBP and DBP, respectively. A numerically larger 12-hour than 24-hour amplitude is seen for both SBP and DBP. The fitted models are shown with the data in Fig. 8 (left), where the circadian variation of BP and HR is aligned with that of the *n*-3 HUFA status. BP and HR data collected around the clock with the same ambulatory monitor at similar about 30-minute intervals on other Sundays in 2009 were similarly analyzed. Analyses are restricted to Sundays in view of differences found earlier between week days and weekends, the study day being also a Sunday. In all cases (33 days), the 24-hour component was detected with statistical significance for SBP and HR, and for DBP, it was found on 29 of the 33 days. In the majority of cases, the 24-hour component was the most prominent in the spectrum (SBP: 30/33; DBP: 23/33; HR: 31/33). A 12-hour prominence was only seen in 1 and 5 days for SBP and DBP, respectively, and it did not occur in the case of HR. As seen in Fig. 8 (right), the 24-hour amplitudes of SBP and DBP on the day of study lie outside the 95% prediction

interval computed on the basis of the results from the other 33 Sundays, whereas the 24-hour amplitude of HR lies at the lower limit of the corresponding 95% prediction interval.

Despite the relatively small number of samples obtained by finger pricking, a circadian rhythm could be demonstrated for 65% of the fatty acid related variables after the data from F and G were expressed as a percentage of their respective 24-hour mean values and pooled. The gain in statistical power to demonstrate a time effect obtained by using relative rather than original data was already shown in 1953 in a study of endogenous eosinopenia in institutionalized patients with mental deficiency (Halberg et al. 1953).

Long-chain PUFAs, especially of the Omega-3 series, in blood lipids are mainly present in relatively stable pools (i.e., the glycerophospholipids), in turn incorporated in the lipoproteins and in circulating cells (mainly erythrocytes' membranes). The various lipid classes are differently distributed in the lipoproteins, and have different profiles of long-chain PUFAs (Risé et al. 2007). Furthermore, even after intakes of appreciable amounts, increments of these fatty acids in blood are rather slow and limited, and, in addition, they are not actively utilized for energy purposes, at variance with shorter-chain saturated and mono-unsaturated fatty acids. It can therefore be proposed that the observed circadian variations cannot be attributed to changes in the balance between intakes and rates of utilization (considering also that there was no ingestion of Omega-3 PUFA during the time of the study), but rather to circadian variations in the pools of fatty acid transporters (triglycerides, phospholipids and cholesterol esters in the different lipoproteins, VLDL, LDL, HDL and chylomicrons). Circadian variations of more labile lipid pools (i.e., triglycerides; Haus and Touitou 1992), rich in oleic acid, may also account for the changes in the opposite direction of long-chain PUFA and oleic acid.

The assessment of the circadian variation in plasma concentrations of major lipoproteins will be a useful addition in future studies.

Apart from the relevance of the *n*-3 status, the absolute values of *n*-3 also need proper consideration in their own right. Indeed, a low *n*-3 status may be associated with low *n*-3 fatty acid values and this is not ideal. The *n*-3 values of F and G are lower than those of an Italian reference population (Table 3), suggesting the desirability of *n*-3 supplementation.

Evidence for keeping current *n*-6 fatty acid intake as high as presently recommended is lacking. The current view is to guarantee a certain amount of long-chain *n*-3 PUFA intake (>200 mg/day), with no need for more than 2–3 energy percent of linolenic acid. Official positions also differ among researchers, as apparent from discussions at various International Congresses on fatty acids and lipids such as the one (7th Congress of the International Society for the Study of Fatty Acids and Lipids, ISSFAL) held in June 2006 in Cairns, Australia. There, a whole morning was devoted to a session entitled "Consensus and Controversies", in which two opposing groups of experts presented evidence in favor of or against the concept of linolenic-acid-rich diets. Presented as problematic was the fact that under current conditions of saturation of most if not all fatty acids metabolic pathways by linolenic acid, the *n*-6 series of fatty acids – including linoleic (LA, 18:2) and arachidonic (AA, 20:4) acids – markedly incorporate in lipid pools, resulting into low incorporation of long-chain *n*-3 fatty acids. On the other hand, the *n*-3 series of fatty acids mainly consist of the long-chain PUFA, eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA). Therefore, to include LA in the total *n*-6, and to consider the total *n*-6 in the calculation of the *n*-3 status may not be appropriate. A preferred way to proceed may be to consider the *n*-3 HUFA index, a marker of the *n*-3 HUFA status [Total *n*-3 HUFA/(Total *n*-6 HUFA + Total *n*-3 HUFA) = 100 × (EPA+DPA+DHA)/(20:3+20:4AA+22:4+22:5+ EPA+DPA+DHA)]. In this case, neither LA nor α-linolenic acid (ALA) is taken into consideration and only the fatty acids containing more than 20 carbons (that are biologically more relevant) are considered.

Since the determination of fatty acids may depend on prior food intake (Dewailly et al. 1981, Kessler Cella et al. 1995b, Romon et al. 1997, Risé et al. 2007, Astorg et al. 2008), the study should be extended to conditions of equidistant, isocaloric meals consisting of the same composition in fatty acids. The aim of the present study, however, was the assessment of the dynamics under ordinary living conditions. It should be noted that on the day of study, F and G had their meals soon after rather than before blood sampling. For reasons noted at the outset (Brillat-Savarin 1826, Chossat 1843, Ågren et al. 1931, Higgins et al. 1932, 1933, Haus and Halberg 1966, Nelson et al. 1973, Halberg et al. 1995), it seems unlikely that the demonstration of a circadian rhythm in the majority of the fatty acids related variables determined depended solely on meals. Indeed, complete bed-rest for 36 hours and a 4-hourly hypo-caloric diet did not abolish the circadian rhythmicity of physiological functions such as SBP, HR, or the urinary excretion of 17-hydroxycorticosteroids, potassium, adrenaline, noradrenaline, and vanillyilmandelic acid in another

study (Reinberg et al. 1970). Similar results were obtained in another bed-rest study for BP (Halberg et al. 1988).

These observations prompt questions to their degree of generality, to secure clinical significance, apart from mere statistical significance (Cornélissen et al. 1994), and to inquire about putative underlying mechanisms and possible implications in research and practice. This may be done in the context of criticism of still debated relationships between cholesterol and cardiovascular disease, in the light of outcomes in the Framingham study, discussed in a broader view by Rosch (2001, 2008), Ravnskov et al. (2006), Ravnskov (2009) and Simopoulos (2009). Studies by Keys (1970) and their follow-ups (Kromhout et al. 2002, Menotti et al. 2003, 2004a, b, 2007, 2008) are pertinent. But in all the discussions, whether it is the role of *n*-3 fatty acids or that of cholesterol, the dynamics as yet need clarification (Smolensky et al. 1972, Singh et al. 2003).

A long series of circadian studies of cholesterol discussed by Dell'Acqua and Gambassi (1960) already received follow-ups (Singh et al. 1989, 1992). In India, population circadian rhythms in total, high-density lipoprotein and low-density lipoprotein cholesterol were reported in small groups of men and women 20–25 years of age, sampled every 8 hours for 24 hours (3 samples) under both usual and fasting conditions (Singh et al. 1989). By contrast, in Italian data from 20 men and women 15–47 years of age collected at 3- to 4-hour intervals for 24 hours (7 samples) (Lippi and Argiolas 1950), a circadian rhythm in cholesterol could not be demonstrated with statistical significance. Instead, 12-hour and 8-hour components accounted for 37.4% and 41.3% of the overall variance, on the average. In North American men and women (N = 23, 71 ± 5 years of age) and in Romanian boys and girls (N = 194, 11 ± 2 years of age), adults (N = 40, 21 ± 2 years of age) and elderly (N = 194, 76 ± 8 years of age) of both genders, but not in English men (N = 20, 22 ± 3 years of age), a circadian rhythm in cholesterol was shown to peak around noon (Haus and Touitou 1992), followed about 6 hours later by triglycerides (Haus and Touitou 1992). Serum concentrations of apolipoprotein, cholesterol and triglycerides were also reported to follow a circadian variation in 25 apparently healthy adults of both genders (29.5 ± 3.6 years of age), the maximal daily variation (with respect to the daily mean) ranging from 5% to 63% (Rivera-Coll et al. 1994). Cholesterol synthesis determined in 5 normolipemic men (22–25 years of age) was also reported to be circadian periodic, assuming near-zero values in the morning and maximal values around midnight (Kessler Cella et al. 1995a).

This fledging start of fatty acids dynamics is presented with the indication that only cost considerations prompted the sampling limited to a single 24-hour span and the pooling of data from two adults of similar age, ethnicity and lifestyle but different gender and medical history. This report is no more than an incentive for further work and in no way does it permit generalization. This feasibility study was carried out in preparation for a larger study planned to examine not only the circadian changes in fatty acids in different age groups, but to extend sampling along the scales of the week, the seasons, and beyond, with added focus on effects of fatty acids on behavior and mental function (Wilczynska-Kwiatek et al. 2010). It will then become possible to assess the relative prominence of circadians versus other anticipated components, including non-photic ones present in the cosmos, which have already been documented to characterize BP and HR (Cornélissen et al. 2007), melatonin (Cornélissen et al. 2008b), breakdown products of steroids (Halberg et al. 2008a), and a host of other variables, including the incidence patterns of mortality from different causes (Cornélissen et al. 2008a, Halberg et al. 2008b).

The planned larger study will have an opportunity to assess the merits of different aspects of the dynamics of fatty acids in the light of various outcome measures, such as the left ventricular mass index, total, low-density lipoprotein and high-density lipoprotein cholesterol, triglycerides, and C-reactive protein, and their dynamics. Long-term follow-up may provide additional information on actual outcomes in terms of morbidity and/or mortality for a further association with the presence or absence of various vascular variability disorders to be assessed from concomitant ambulatory BP and HR monitoring interpreted chronobiologically. The relative merits of fatty acids versus cholesterol as biomarkers may thus gain clarification. A precedent related to BP and HR monitoring already unveiled vascular variability disorders not screened for in current medical practice today that are associated with an increase in cardiovascular disease risk as large as if not larger than the risk of an elevated BP (Singh et al. 2003, Sánchez de la Peña 2008, Halberg et al. 2009).

CONCLUSION

Since the incidence of vascular variability disorders was found to increase in association with pre-hypertension and pre-diabetes, it will be important to see whether any outcome-based

undesired patterns related to the fatty acids and/or to other lipids, notably cholesterol and triglycerides, may similarly point toward a pre-metabolic syndrome, so that dietary countermeasures may be accordingly designed.

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