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Phosholipid trafficking between serum-HDL-erythrocyte in various conditions associated with circadian disturbances

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Abstract: Circadian disturbances are known to affect lipid metabolism. Most of the phospholipid metabolites have been shown to be under circaian control by metabolomics studies. Moreover, genes related to glycerolipid synthesis were reported to be circadianly regulated. Data from various studies revealed a relationship between glycerolipid metabolism and circadian misalignments. Some proteins responsible for transfer of phospholipids among plasma lipoproteins or membranes include phospholipid transfer proteins (PLTP), lecithin cholesterol acyltransferase (LCAT), secretory phospholipase A2 (sPLA2), endothelial lipase (EL), exhibits circadian oscillation. However no data are available as to whether circadian disturbances can influence phospholipid trafficking among HDL, erythrocytes and serum. To this end, , four conditions associated with circadian disturbances including type 2 diabetes, prediabetes with metformin usage, psoriasis and night-shift work were investigated for phospholipid trafficking. Indices of circadian misalignments, plasma melatonin and cortisol levels, were determined by ELISA and chemiluminescence methods respectively. Serum levels of PLTP, LCAT, EL and sPLA2 levels were analyzed by ELISA. Phospholipid compositions were investigated by two-dimensional HPTLC and/or HPLC.
Results by HPLC indicated that PE/PC ratios in erythrocyte lysates of diabetes and metformin groups were found to be significantly lower compared to that of controls which might be associated with the lower levels of LCAT, EL and PLTP levels measured. Altered plasma melatonin levels indicated circadian misalignments in these conditions. However, in psoriasis and night-shift groups, circadian indexes did not match with the PE/PC ratios in erythrocytes as it was in diabetes and metformin groups. We therefore conclude that circadian as well as metabolic disturbances both might have a role in phospholipid trafficking.

Keywords : Chronobiology disorders; phospholipids; erythrocyte membrane; phospholipid transfer proteins; LCAT protein; LIPG protein; Phospholipases A2, secretory.

1. INTRODUCTION

Desynchronization between environmental and endogen circadian cues is known to cause circadian peturbations. Continual access to food and exposure to lights as well as night shift working conditions , traveling between different time zones , which are commonly encountered in modern life styles all lead to disturbances in circadian physiology. Moreover, some of the drugs used , consumption of high fat diets, and sleep restrictions have also been reported to interfere with biological rhythms and consequently bring about

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various metabolic dysfunctions. Various previous works indicate that circadian disorders can provoke endocrine [1] and menthal illnesses [2], metabolic syndrome [3,4], neuropsychiatric disorders [5] and cardiovascular diseases [6].

Studies on circadian perturbances by sleep restrictions point out alterations in lipid metabolism. Under sleep restricted conditions , diacylglycerol levels were found to be decreased whereas phospholipids were increased in both human and rat plasma. Phosphatidylcholine, lysophosphatidylcholine, phosphatidylethanoamine, and sphingomyeline were among the phospholipids increased most in the blood [7]. Metabolomics studies conducted on healthy subjects demonstrate that blood levels of these lipids are under circadian control and exhibit daily oscillations [8]. Genes related to glycerolipid synthesis and phospholipases were reported to be among the circadianly regulated mammalian genes [9,10]. Data from various studies reveal a relationship between glycerophospholipid metabolism and circadian misalignments. Since outer layer of cell membrane is mostly composed of the phospholipids , alterations in phospholipids by circadian misalignments might result in changes in membrane structure and function with possible consequences in signal transduction through membrane receptors [11]. Among the proteins responsible for the production of various phospholipid metabolites and for transfer of phospholipids among plasma lipoproteins including HDL, VLDL and LDL are phospholipid transfer proteins (PLTP) , lecithin cholesterol acyltransferase (LCAT) , secretory phospholipase A2 (sPLA2), endothelial lipase (EL). Since *Lcat, Lipg and Pltp* are rhythmically expressed [9,10], shifted circadian oscillation of these gene might cause alteration in phospholipid homeostasis. However, no data available as to how circadian disturbances can influence phospholipid trafficking among HDL-erythrocytes and serum. To this end, four conditions associated with circadian disturbances including type 2 diabetes [12], prediabetes with metformin usage [13], psoriasis [14] and night shift working [15] were investigated for phospholipid trafficking between serum-HDL-erythrocyte lysates.

2. RESULTS

2.1. Demographic characteristics of study subjects: The demographic information of the experimental and control groups is presented in Table 1. The avarage age of the control group subjects was 38.5 years old .Whereas that of diabetes and metformin group subjects were found to be moderately higher (50 and 48 respectively) compared to that of control subjects. BMI of both diabetes and metformin groups were also found to be significantly higher. Majority of subjects in groups were males except for metformin group subjects. Percentage of subjects being current smokers was similar between groups.

2.2. Biochemical analyses of blood glucose and lipid profile : Table 2 depicts serum biochemical parameters of subjects in each group. Serum fasting glucose levels of subjects in both diabetes and metformin groups were found to be significantly higher as compared to that of control group. On the other hand serum average triacylglycerol and VLDL-C concentrations of night-shift group were found to be elevated with respect to other groups. .

2.3. Serum melatonin and cortisol levels as biomarkers of circadian rhythm : Table 3 illustrates serum melatonin and cortisol levels. In terms of melatonin, diabetes and metformin groups exhibited significantly lower levels, whereas night shift group higher levels as compared to that of control.Intergroup comparisons revealed that diabetes and metformin groups have lower levels compared to psoriasis and night-shift group. On the other hand, cortisol level was found to be significantly higher in night-shift group as compared to that of control. Cortisol level of psoriasis was lower compared to diabet and night-shift group, wheras that of metformin was lower compared to night-shift group. Present results imply circadian phase shifts in diabet, metformin and night-shift groups. It can also be envisaged that night-shift group being antiphase with diabet and metformin groups.

2.4. Levels of proteins (PLA_2, LCAT, EL, PLTP) related to phospholipid trafficking :
Analysis of serum LCAT, EL and PLTP by ELISA revealed lower levels in diabetes and metformin groups compared to control (Table 4). The same also applies to psoriasis group except for EL. In terms of sPLA2, none of the groups exhibited a significant variation from the control group. Night-shift group exhibited significantly higher LCAT levels compared to diabetes and metformin group and higher EL levels compared to diabetes group. Diabet and metformin affected LCAT, EL and PLTP levels most . Wheras night-shift group were affected least. PLTP was the only protein , level of which was significantly altered by night-shift compared to that of controls. In general it can be envisaged that the conditions associated with circadian disturbances lower phospholipid trafficking activity.

2.5. Phospholipid compositions of serum, HDL, and erythrocyte lysates

Phospholipids of the extracted samples were separated by two-dimensional HPTLC .Consequently, lipid bands were visualized by staining and scanned. The densities of lipid spots were densitometrically analyzed. Ratios of densitometric raw volumes for each of the phospholipid classes in erythrocyte lysates to that in HDL fraction, erythrocyte lysates to that in serum, or in serum to that in HDL fraction were depicted in Table 5. The ratios were compared among control and experimental groups. No significant differences were found. However, The ratio of LPC level in serum to that in HDL fraction appeared to be relatively higher, although insignificant, in psoriasis and shift worker groups compared to those in other groups which might imply release of lysophosphatidylcholin from phosphatidylcholin of HDL to serum by the action of sPLA2 , LCAT or both. PE/PC ratios in erythrocyte lysates, serum or HDL fraction in either groups were also compared (Table 5). Comparison of the ratios among the groups indicated insignificant correlation.

Some of the aliquots of the extracted samples were allocated to HPLC analyses and the phospholipid classes were detected by a UV detector at 203 nm. Ratios of detector responses as mAU for each of the phospholipid fractions in erythrocyte lysate to that of the corresponding one in HDL fraction, that in erythrocyte lysate to that in serum, or that in serum to that in HDL fraction were determined and the results were exhibited in Table 6. PE/PC ratios in erythrocyte lysates, serum or HDL fraction in either groups were also compared (Table 6.). PE/PC ratios in erythrocyte lysates of diabetes, metformin as well as psoriasis groups were found to be significantly lower compared to that of control and shift-work group. In terms of ratios of PE or PC levels either in erythrocyte lysate to HDL, erythrocyte lysate to serum, or serum to HDL, comparison of differences among groups produced insignificant results.

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Table 1. Some demographic information of control and patient groups

Variables **		Groups*										
		n	Control	n	Diabetes Mellitus	n	Metformin	n	Psoriasis	n	Shift worker	
			Median(min-max)		Median(min-max)		Median(min-max)		Median(min-max)		Median(min-max)	*p*
Age		26	38.5(25-51)[b,c]	23	50(29-67) [a,d,e]	23	48(20-76) [a,d,e]	25	31(18-71)[b,c,e]	25	40(33-55)[b,c,d]	<0.001*
BMI		26	26.2(22.1-34.5)[b,c]	20	30.2(21.5-40.5)[a,d,e]	23	30.1(20.9-39.2) [a,d,e]	25	25.5(18.4-38.7)[b,c]	25	26.8(17.9-33.7)[b,c]	0.002*
		n	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	***p***
Smoking status	**Current**	9	34.6	10	47.6	8	34.8	11	44	15	60	0.351
	Never	17	65.4	11	52.4	15	65.2	14	56	10	40	
Gender	**Male**	15	57.7	18	78.3	11	47.8	10	40 [e]	20	80 [d]	0.011*
	Female	11	42.3	5	21.7	12	52.2	15	60 [e]	5	20 [d]	
Medication	**User**	0	0 [a]	6	26.1 [a]	23	100 [b]	7	28 [a]	7	28 [a]	<0,001*
	Nonuser	26	100 [a]	17	73.9 [a]	0	0 [b]	18	72 [a]	18	72[a]	

BMI: Body Mass Index, a: Different compared to the control group, b: Different compared to the diabetes Mellitus group, c: Different compared to the metformin group, d: Different compared to the psoriasis group, e: Different compared to the shift worker group. ** Variables are summarized as median (min.-max.), $p<0.05$.

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Table 2. Serum biochemical parameters of control and patient groups

Variables**	Groups										
	Control		Diabetes Mellitus		Metformin		Psoriasis		Shift worker		
	n	Median (min-max)	n	Median (min-max)	n	Median (min-max)	n	Median (min-max)	n	Median (min-max)	*p*
Fasting Glucose (mg/dL)	23	90(82-101)[b,c]	19	163(74-350)[a,c,d,e]	22	114.5(85-188)[a,b,d,e]	17	93(78-194)[b,c]	20	95(77-112)[b,c]	<0.001
HDL-C(mg/dL)	26	42.35(33-69.9)[b,e]	23	37.9(28.4-63.2)[a,d]	23	42.2(26.8-79.2)	25	43.6(30.6-66.9)[b,e]	25	37.2(24.4-74.1)[a,d]	0.025
LDL-C (mg/dL)	26	114.2(74.3-167.2)	20	119.15(49.7-232.7)	23	134.4(59.3-195.1)	23	109.3(54.8-180.7)	24	104.05(64.8-199.1)	0.307
VLDL-C(mg/dL)	26	24.5(9-76.4)[e]	20	33(11.4-127.8)[d]	23	30.8(14.2-62)[d]	23	22.4(5.8-50)[b,c,e]	24	36(10.2-109.4)[a,d]	0.010
Total C (mg/dL)	26	196(130-239)	21	188(142-304)	23	204(118-295)	23	172(104-265)	24	181(149-268)	0.152
TAG (mg/dL)	26	123(45-382)[e]	20	165(57-639)[d]	23	154(71-310)[d]	23	112(29-250)[b,c,e]	24	180(51-547)[a,d]	0.009

HDL-C: High Density Lipoprotein-Cholesterol, VLDL-C: Very Low Density Lipoprotein-Cholesterol, LDL-C: Low Density Lipoprotein-Cholesterol, TAG: Triacylglycerol, C: Cholesterol, a: Different compared to the control group, b: Different compared to the Diabetes Mellitus group, c: Different compared to the metformin group, d: Different compared to the psoriasis group, e: Different compared to the shift worker group. ** Variables are summarized as median (min.-max.), $p<0.05$.

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Table 3. Serum melatonin and cortisol levels of control and patient groups

Variables **	Groups *										
	Control		Diabetes Mellitus		Metformin		Psoriasis		Shift worker		
	n	Median (min.-max)	n	Median (min.-max)	n	Median (min.-max)	n	Median (min.-max)	n	Median (min.-max)	*p*
Melatonin (ng/L)	20	42.5 (5.86-135.26)^{b,c,e}	22	19.64 (6.15-39.07)^{a,d,e}	21	18.78 (8.26-62.75)^{a,d,e}	24	39.17 (0.38-144.92)^{b,c,e}	19	69.36 (33.03-780.01)^{a,b,c,d}	<0.001
Cortisol (µg/dL)	26	Kas.33 (7.17-20.59)^{e}	23	Ara.83 (6.47-20.21)^{d}	23	Kas.39 (1.5-19.66)^{e}	25	Eyl.99 (2.24-19.22)^{b,e}	25	15.84 (6.74-24.14)^{a,c,d}	0.003

a: Different compared to the control group, b: Different compared to the Diabetes Mellitus group, c: Different compared to the metformin group, d: Different compared to the psoriasis group, e: Different compared to the shift worker group. ** Variables are summarized as median (min.-max.), $p<0.05$.

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Table 4. Serum sPLA2, LCAT, EL and PLTP levels of control and patient groups

Variables **	Groups *											
		Control		Diabetes Mellitus		Metformin		Psoriasis		Shift worker		
	n	Median (min.-max)	n	Median (min.-max)	n	Median (min.-max)	n	Median (min.-max)	n	Median (min.-max)	*p*
$sPLA_2$ (ng/L)	22	507.45 (196.19-2889.13)	23	447.63 (248.32-668.85)	23	453.83 (265.39-1552.26)	25	527.72 (258.51-1205.17)	23	637.53 (300.38-3375.15)	0.066
LCAT (ng/L)	20	4567.08 (1230.2-9226.07)^{b,c,d}	22	1647.43 (269.33-4937.23)^{a,d,e}	23	1646.5 (217.15-9816.28)^{a,e}	25	2248.82 (419.87-8955.8)^{a,b}	21	3709.4 (1425.8-6698.2)^{b,c}	0.001
EL (ng/L)	24	491.38 (218.79-2792.26^{b,c}	23	289.57 (190.13-678.13)^{a,e}	23	301.82 (245.25-880.2)^{a}	25	350.72 (190.13-602.51)	22	363.39 (245.46-2377.41)^{b}	0.018
PLTP (ng/L)	17	1763.32 (779.6-5949.1)^{b,c,d,e}	20	865.49 (188.92-3760.07)^{a}	19	804.1 (303.27-3316.77)^{a}	24	771.84 (104.38-3770.48)^{a}	20	1066.13 (131.48-5384.18)^{a}	0.006

sPLA2: Secretory phospholipase A2, LCAT: Lecithin cholesterol acyltransferase, EL: Endothelial lipase PLTP: Phospholipid transfer proteins. a: It is different compared to the control group, b: It is different compared to the diabetes group, c: It is different compared to the metformin group, d: It is different compared to the psoriasis group, e: It is different compared to the shift worker group. ** Variables are summarized as median (min.-max.), $p<0.05$.

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Table 5. Phospholipid ratios in serum, erythrocyte lysate and HDL fraction by HPTLC

Variables**		Groups					
		Control (n=13)	Diabetes Mellitus (n=10)	Metformin (n=11)	Psoriasis (n=14)	Shift worker (n=14)	
		Median (min.-max)	Median (min.-max)	Median (min.-max)	Median (min.-max)	Median (min.-max)	*p*
E/H	PE	2.70 (1.46-4.33)	3.03 (1.36-5.01)	2.93 (1.36-5.36)	3.55 (1.54-4.86)	3.27 (1.77-8.12)	0.443
	PC	0.89 (0.47-1.36)	0.83 (0.56-1.23)	0.98 (0.72-1.62)	0.82 (0.63-1.56)	1.01 (0.37-1.45)	0.380
	SM	1.50 (0.87-2.73)	1.41 (1.06-1.86)	1.57 (1.22-2.59)	1.41 (0.99-3.9)	1.86 (0.75-2.69)	0.108
	PS+PI	2.64 (1.23-4.75)	2.35 (0.46-4.1)	2.75 (1.88-6.63)	2.72 (1.62-6.71)	2.83 (1.09-6.74)	0.574
E/S	PE	2.66 (1.37-3.89)	2.38 (1.51-6.68)	2.87 (1.49-4.58)	3.04 (1.37-4.40)	2.5 (1.32-5.27)	0.922
	PC	0.77±0.21	0.72±0.16	0.93±0.35	0.81±0.24	0.75±0.23	0.303
	SM	1.25±0.28	1.15±0.32	1.56±0.7	1.18±0.27	1.29±0.4	0.464
	PS+PI	2.78±0.83	2.46±1.48	2.96±1.42	2.5±0.74	2.89±1.38	0.786
S/H	PE	1.10±0.36	1.09±0.32	1.1±0.49	1.23±0.41	1.39±0.48	0.299
	PC	1.24±0.44	1.21±0.3	1.32±0.63	1.18±0.3	1.37±0.48	0.766
	SM	1.40±0.44	1.3±0.35	1.25±0.48	1.32±0.39	1.45±0.46	0.766
	PS+PI	0.98 (0.56-1.83)	0.91 (0.44-2.06)	1.04 (0.52-2.17)	1.18 (0.79-2.40)	1.21 (0.54-3.27)	0.455
	LPC	0.86 (0.43-1.73)	0.84 (0.58-1.43)	0.86 (0.40-1.86)	1.04 (0.80-1.78)	1.04 (0.50-1.91)	0.075
E	PE/PC	1.08±0.23	1.08±0.15	1±0.28	1.08±0.2	1.02±0.24	0.846
S	PE/PC	0.32±0.08	0.3±0.11	0.3±0.05	0.31±0.07	0.28±0.06	0.779
H	PE/PC	0.34 (0.23-0.61)	0.31 (0.26-0.42)	0.36 (0.25-0.44)	0.29 (0.19-0.61)	0.27 (0.15-0.40)	0.058

E: Erythrocyte, S: Serum, H: HDL, PE: Phosphatidylethanolamine, PC: Phosphatidylcholine, PS: Phosphatidylserine, PI: Phosphatidylinositol, SM; Sphingomyelin, LPC: lyso- Phosphatidylcholine. ** Variables are summarized as median (min.-max.) or mean ±standard deviation $p<0.05$.

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Table 6. Phospholipid ratios in serum, erythrocyte lysate and HDL fraction by HPLC

Variables**		Groups					
		Control (n=6)	**Diabetes Mellitus (n=6)**	**Metformin (n=6)**	**Psoriasis (n=6)**	**Shift worker (n=6)**	
		Median (min.-max)	**Median (min.-max)**	**Median (min.-max)**	**Median (min.-max)**	**Median (min.-max)**	***p***
E/H	**PE**	2.34 (1.63-2.81)	3.09 (1.66-16.13)	3.41 (1.49-9.43)	3.06 (1.80-15.93)	4.06 (2.08-8.41)	0.329
	PC	1.08 (0.57-1.37)	1.07 (0.92-9.80)	1.55 (0.57-2.14)	1.15 (0.93-1.66)	1.29 (0.95-2.32)	0.378
E/S	**PE**	7.94 (2.83-13.95)	5.59 (2.93-11.13)	8.81 (3.99-26.39)	5.84 (4.83-32.80)	11.25 (3.95-19.70)	0.449
	PC	1.92 (0.79-3.14)	1.31 (1.02-2.04)	1.43 (0.51-3.07)	1.39 (1.04-2.04)	1.40 (0.80-2.36)	0.953
S/H	**PE**	0.30 (0.20-0.67)	0.52 (0.34-2.12)	0.37 (0.24-0.48)	0.42 (0.24-1.35)	0.34 (0.17-2.13)	0.145
	PC	0.65 (0.32-0.97)	0.94 (0.62-4.79)	1.17 (0.64-1.46)	0.85 (0.58-1.08)	1.08 (0.48-1.51)	0.225
E	**PE/PC**	0.52[a] (0.44-0.58)	0.49 [a, b] (0.36-0.54)	0.43[b] (0.40-0.47)	0.47 [a, b] (0.42-0.53)	0.52[a] (0.42-0.56)	**0.041***
S	**PE/PC**	0.15 (0.07-0.18)	0.14 (0.04-0.16)	0.06 (0.05-0.09)	0.10 (0.03-0.17)	0.07 (0.05-0.11)	0.064
H	**PE/PC**	0.25 (0.16-0.30)	0.19 (0.12-0.31)	0.18 (0.09-0.28)	0.18 (0.031-0.41)	0.17 (0.08-0.55)	0.548

E: Erythrocyte, S: Serum, H: HDL, PE: Phosphatidylethanolamine, PC: Phosphatidylcholine. * Differences between groups with different superscripts were found to be statistically significant. ** Variables are summarized as median (min.-max.), $p<0.05$.

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3. DISCUSSION

In order to study the phospholipid traffic between serum, HDL, and erythrocyte lysates in patients newly diagnosed as T2DM, prediabetic patients using metformin, psoriatic patients and night shift workers, all of which are known to be concurrent with circadian disturbances, plasma levels of enzymes involved in the phospholipid traffic (PLTP, LCAT, EL and sPLA2) were analysed by ELISA in conjunction with compositional analyses of phospholipids by HPTLC and/or HPLC. Interrelatedness of the variants were analysed by various statistical methods. Since the measures of melatonin as well as cortisol are considered the peripheral indices of human circadian timing based on an internal 24-hour clock [19], plasma levels of both melatonin and cortisol in patients serum were also determined.

In the current work, deviation of melatonin levels from that of controls in diabet-metformin group indicates a prominent circadian disruption which is in line with previous works. Cell autonomous circadian clocks orchestrate the regulation of physiological functions required for maintenance of normal glucose homeostasis. Some investigators pointed out shifted circadian rhytms associated with diabetes and metformin. Pathogenesis of T2DM was reported to be linked with impairments in circadian regulation both at the level of central and peripheral circadian oscillators [20]. Metformin is an orally effective insulin-sensitizing drug widely prescribed for treating T2DM. In mice, metformin was found to affect the circadian clock and metabolic rhythms in a tissue-specific manner. This effect was mainly of phase advances in the liver and phase delays in the muscle in clock and metabolic genes and/or protein expression [13]. Metformin led to mainly high-amplitude shifted circadian rhythms in muscle cells (21).In the present work, as indicators of the circadian rhythm, plasma melatonin and cortisol levels were found to be significantly higher compared to that of the control. These findings indicate circadian misalignment for the subjects of night-shift group which complies with previous data indicating circadian misalignment associated with shift-work condition. Of the many health-related effects of shift work, disrupted sleep is the most common. The reason for the health problems in shift work is the conflict between displaced work hours and the output of the biological clock [22]. The effects of shift work on physiological function through disruption of circadian rhythm are well described. In humans , prolonged sleep restriction with concurrent circadian disruption alters metabolism and could increase the risk of obesity and diabetes [23]. The link between shifted circadian rhythm and metabolic diseases (diabetes) and CVD has been suggested [24]. Prolonged exposure to light in the work environments of shift workers leads to changes in peaks and troughs of melatonin levels. They have poorer quality of sleep [25,26].

There are several reports suggesting that pathophysiology of psoriasis may be associated with aberrant circadian rhythms. Night shift work has been associated with an increased risk of psoriasis co-morbidities, with the major underlying mechanism postulated to be exposure to light during the night leading to disrupted circadian rhythm and decreased melatonin synthesis [27]. Moreover, circadian *Clock* gene regulates psoriasis-like skin inflammation in mice, establishing a mechanistic link between psoriasis and the circadian clock [28]. Psoriatic patients had lost the nocturnal peak and usual circadian rhythm of melatonin secretion [27].However in our work , psoriasis was the only experimental group neither plasma melatonin nor cortisol levels of which significantly deviated from that of controls. This might be because of different circadian metabolic phenotypes in humans [29]. Variation in type and severity of the psoriasis might also have impeded to find out any shift in the level of these circadian rhythm indices.

LCAT is mostly found attached to HDL and esterifies cholesterol and forming lysophosphatidylcholine from phosphatidylcholine. Activity of LCAT is known to raise HDL-C level . In LCAT deficiency, HDL-C was reported to be decreased [30-32]. Various pathological conditions are known to modify LCAT activity which in turn can alter plasma HDL levels. In type 2 diabetes, Apo A-I , main apoprotein of HDL, glycation was reported to decrease its stability and results in shorter half-life of apo A-I [33]. In the current work, LCAT levels were found to be significantly lower in diabetes and metformin groups . The HDL-C levels were also found to be lower for diabetics. Nakhjavani et al [34] reported that LCAT activity was lower among patients with type 2 diabetes compared to subjects without type 2 diabetes and it had a greater reduction in the presence of metabolic syndrom [34,35]. Although LCAT mass was related to LCAT activity, the strength of this association was lower because of errors of the assays [36].

In the present work, PLTP levels of all the patient groups was found to be lower compared to that of control group.Few investigations addressed the issue of PLTP particularly in dibates. Nonetheless results so

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far have been controversial reporting either increased or decreased PLTP activity or concentration. However, increased PLTP activity was attributed to obesity and not necessarily to diabetes [37]. On the other hand, in line with the data from others' workers, HDL-cholesterol levels of diabetes and night-shift groups were significantly lower. PLTP can transfer phospholipids and free cholesterol from the surface of VLDL or chylomicron onto nascent HDL to form mature HDL. PLTP deficiency causes a significant reduction of HDL cholesterol and phospholipid levels in circulation [38,39]. PLTP activity plays an important role in plasma HDL composition [39]. However we did not find any alteration in HDL phospholipid compositions analysed either by HPTLC or HPLC.

EL acts mainly as phospholipase and is more active against HDL and plays a significant role in the HDL metabolism. Contrary to our expectation, plasma EL levels in diabetes and metformin groups were found to be significantly lower compared to that in control. Whereas previous works indicated increased serum EL concentration in type 2 diabetic patients [40]. Usage of anti-diabetic drugs might have caused this discrepancy because exogen insulin therapy is known to lower serum EL concentration [41]. However, animals that are deficient in EL were reported to have a marked elevation in HDL-cholesterol levels [42] which is opposite of our results. But Potocnjak et al pointed out that association between EL and lipid/lipoprotein plasma levels was modulated by metabolic syndrome. EL- and lipid/lipoprotein plasma levels were different in patients with metabolic syndrome compared to those without metabolic syndrome [43]. However, in our work, assesments of subjects of diabet or metformin groups for metabolic syndrome was not made which is one of the limitations of our study.

Role of phospholipase A_2 enzymes in a pathogenic pathway in psoriasis has been suggested [44]. Previous studies have demonstrated increased activity of secreted PLA_2 in psoriatic patients [44]. Besides, inhibition of phospholipases suppress progression of psoriasis through modulation of inflammation [45]. Nevertheless this was not the case in our work which indicated no change in plasma sPLA2 levels. Dyslipidemia is one of the comorbidities in psoriatic patients. Total cholesterol level along with LDL, VLDL, TG and apo B concentrations has been proven to be higher in psoriatic patients. On the other hand, HDL-C concentration is significantly decreased. Total concentrations of phosphatidylethanolamine and phosphatidylcholine were decreased whereas plasma concentration of lysoglycerophospholipids were significantly increased. Some reports, however, do not present any differences in the levels of serum phospholipids between psoratic patients and healthy control group [46-50]. However, in psoriatic group we did not found atherogenic lipid profile, both cholesterol and TG levels were within the normal range . Numerous studies show decreased levels of HDL and/or increased levels of LDL, VLDL and triglycerides in patients with psoriasis. However, some studies failed to demonstrate an association between lipid serum levels and psoriasis [47]. Antipsoratic drugs can be responsible for the lipid profile disturbances in psoratic patients, because of their action on the circulating lipids. Retinoids have the most potent activity on increasing the levels of triglycerides, total cholesterol, LDL cholesterol and VLDL cholesterol and decreasing HDL fraction. Cyclosporin has milder effects on the lipid profile but it can also lead to TG elevation [48].

In contrast to other experimental groups, only PLTP was found to be altered in night-shift group. Besides no difference in phospholipid compositions was evident in either samples tested. Night-shift group can be comparable with sleep restriction. In sleep restricted subjects which is associated with circadian clock disruption, some of the serum lipid level were reported to be quantitatively changed. Elevated phospholipids were noted. Altered metabolites included some PCs, lysoPCs among others (8). In humans, PLs (PC, LPC, PE, SM) were the most elevated as a function of sleep restriction. Membrane breakdown or release from circulating lipoproteins were proposed as possible common sources for the elevated PLs . Elevated LPCs in blood are possibly derived from sPLA2 acting on lipoprotein PC, LCAT acting on LDL or HDL, or EL acting on HDL (8). Nevertheless, none of these enzymes was found to be different in our night-shift group which contained junior as well as senior workers. There are reports pointing out discrepancies between short- and long-term health consequences of sleep disruption [51]. It is therefore possible that some metabolic adaptations might have occured in senior workers resulting in different values ,and as a result, different group averages.

Our HPLC results indicated that erythrocyte PE/PC ratio was lower in diabetes and metformin group which is in line with the work of Chang et al [52]. Although PE/PC ratio in serum and HDL were also lower compared to that of control, the differences were found to be statistically insignificant. It can be envisaged that higher phosphatidylcholine level might have resulted because of low LCAT levels which is reflected in the decreased ratio. Lower HDL-C levels in diabetics can also be accounted by low LCAT levels. On the contrary to our expectation however , we cound not find any difference in the ratio of LPC level of serum to that of HDL fraction probably because of relatively high variations and the limitations in sensitivity of HPLTC method. Besides, alteration in both EL and PLTP levels can alter PE/PC ratios (9). Since *Lcat, Lipg and Pltp* are rhythmically expressed (9,10), shifted circadian oscillation of these gene might have caused

alteration in PE/PC ratios .On the other hand, the effect of circadian misalignment on PE-methylation pathway (metylation of PE to PC) which was reported to be under diurnal regulation [53] could be an another factor which should be considered . However metabolic alterations occuring in diabetes as well as in psoriasis can not be excluded as possible factors influencing the phospholipid composition.

Some studies point out association between phospholipids and insulin resistance . It was reported that fasting plasma insulin and HOMA-IR were positively correlated with erythrocyte membrane PE and PC content. Moreover, in patients with T2DM and T1DM , it was reported that acidic phospholipids (PI and SM) increased, whereas neutral ones (PE and PC) decreased [54]. On the other hand, skeletal muscle PC/PE ratio was elevated in type 2 diabetic patients compared with obese patients. Basal PC/PE ratio was negatively related to insulin sensitivity among all participants. The relationship between insulin sensitivity and PC or PE content in experimental studies is vague. Whether changes in PC and PE content play a key role in insulin sensitivity awaits further research [52].

In our HPLC results, lower erythrocyte PE/PC ratio was also observed in psoriasis group. In psoriatic patients ,on the other hand, a decrease of concentration of total phospholipids , as well as PE, PC, and the PC:cholesterol ratio in the serum was reported by other workers. There was also an increased level of LPC. Some reports, however, do not present any differences in the level of serum phospholipids between psoriatic patients and healthy control group [46-50]. Since our results indicate no circadian shift in psoriasis group, alteration in PE/PC of erythrocytes can not be attributed to circadian disturbances . Contrary to our expectation , although our circadian indices clearly indicated circadian misalignments , we were unable to detect alteration in PE/PC ratio of erythrocytes in night-shift group. It is therefore can be ascribed to metabolic disturbances, rather than circadian misalignment associated with this condition.

Levels of LCAT, EL and PLTP are all important modulators HDL and/or HDL-cholesterol. Therefore, lower levels of these proteins determined in various conditions tested in the current work might have been reflected in lower HDL-cholesterol levels in diabetes and night-shift groups. Lower plasma LCAT, EL and PLTP activities can all alter phospholipid composition (PC and/or PE) of serum, HDL or cell membranes (9). Phospholipid composition of eryhtrocyte membranes has been reported to vary in various patologies. In metabolic syndrome, lipid analysis of erythrocyte membranes revealed reduced content of phospholipids, SM and PC coupled to increased content of cholesterol, LPC, PS and PE [55].

PC and PE are the most abundant phospholipids in all cell membranes. Abnormally high, and abnormally low, cellular PC/PE molar ratios in various tissues can influence energy metabolism and have been linked to disease progression. Changes in the PC and/or PE content of various tissues are implicated in metabolic disorders such as atherosclerosis, insulin resistance nonalcoholic fatty liver disease and obesity [56].

4. CONCLUSION

The circadian clock influences nearly all aspects of metabolism.Studies established a key role for the molecular circadian clock in regulating lipid homeostasis. This is achieved through rythmic activation and repression of clock-controlled metabolic genes. Lipid pathways are under circadian control in all of the major metabolic tissues , and loss of clock function results in abnormal fat storage and lipid transport, and TAG levels and deficits in absorption of dietary lipids [57]. *Lcat, Lipg and Pltp* are reported to be rhythmically expressed (9,10). As peripheral indices of human circadian timing , plasma melatonin levels, indicates circadian disruption in all the conditions except for psoriasis tested in the current work. it could be envisaged that circadian disturbances might cause alteration in phospholipid related metabolism and as a result in membrane phospholipid compositions. Wether the alteration in membrane phospholipid composition is due to circadian misalignments or metabolic disorders associated with the diabetes or psoriasis, in both of which circadian indices indicated misalignment, is currently unclear. Although the link between shifted circadian rhythm and concurrent alterations in metabolism has been shown by many reports, this still awaits further detailed work. Whether or not alteration in PE/PC ratio in erythrocyte membrane can be used for the diagnosis of circadian misalignment or what role this has in the pathogenesis of circadian rhythm disorder related diseases remain to be seen.

5. MATERIALS AND METHODS

5.1. Subjects and experimental design : The procedure were conducted in accordance with the guideline set by İnönü University, Malatya Clinical Research Ethical Committee (Protocol number : 2019/143). For detecting a 20% difference between diseased and non-diseased groups at 95% confidence level (α=0,05) and 80%power (β=0.020), the required minimum sample size was calculated as 22 per group. The participants were assigned to 5 groups , four experimental and one control group. The patient group aging between 18-71

consisted of 25 subjects with psoriasis, 25 night shift workers, 23 patients newly diagnosed as type II Diabetes Mellitus and 23 pre-diabetic patients using metformin.The control group included 26 age interval-matched subjects.

5.2. Preparation of erythrocyte lysates and lipid extraction : Blood samples were taken into tubes containing EDTA and centrifuged at 1500 g for 10 min at 15°C. At the end of centrifugation, plasma and buffy coat were the discarded, 0.1 mL of the remaining erythrocytes was taken into each Eppendorf tube and 1.9 mL of PBS solution was added. It was vortexed for 10 seconds. After centrifugation in a microcentrifuge at 15000 rpm for 15 minutes at 0 °C, the upper phase was separated and discarded. 1.9 mL of PBS solution was added to each Eppendorf tube again. It was vortexed for 10 seconds. After centrifugation in a microcentrifuge at 15000 rpm for 15 minutes at 0 °C, the upper phase was separated and discarded (16). Having discarded the supernatant , the lower fraction was taken into Eppendorf tubes containing 1 mg of BHT (2,6-di-tert-butyl-4-hydroxytoluene) and equal volume of saline was added and vortexed. Then the resultant erythrocyte suspensions were kept at -80°C until analyzed. The method of Bligh and Dyer (17) was used for the lipid extraction of 1 mL of the erythrocyte lysates. The final extracts were evaporated under nitrogen and resuspended in 0.2 mL of chloroform and kept at 4°C until analyzed.

5.3. HDL separation: Isolation of serum HDL was performed by Warnick et al's method (18) which depends on precipitation of serum lipoproteins (VLDL, IDL, LDL) by polyanion and divalent cations.

5.4. Separation of phospholipids by HPLC : The HPLC equipped with Diode Array Detector (DAD) used in this study was a Agilent 1100. The extracted lipids was dissolved in a mobile phase solvent before HPLC analysis. Separation of phospholipids were conducted on a silica gel column (Develosil 60 A, 250x4.6 mm i.d., with particle size of 5µm, S1005546250W, Japan) by isocratic elution with acetonitrile-methanol-phosphoric acid (130:5:1.5, v/v/v). The sample volume injected for HPLC analysis was 20 µL. The phospholipids were monitored at 203 nm.

5.5. Two dimensional HPTLC for phospholipids : The 20x10 cm silica gel G60 HPTLC plates were activated for 1 hr at 120 °C. The lipid extract was applied to the origin. The solvent for the first direction was chloroform-methanol-25% ammonium hydroxide (90:54:11, v/v). After development in the first dimension, the plates were dried for 15 min in an air current. Then the plates were developed in the second direction in the solvent composed of chloroform-methanol-acetic acid-water (90:40:12:2, v/v).The lipid bands were visualized by spraying with an aqueous solution of 10% copper (II) sulphate in 8% phosphoric acid and subsequent charring at 180°C for 20 mins. The plates were scanned by a scanner (Canon 5600F) .The densities of lipid spots were densitometrically analyzed by a software (Sweday Just TLC, Sweden) . The densitometric raw volumes determined was used as a measure of spot signal intensity .

5.6. ELISA analyses: ELISA analyses were conducted for detecting melatonin (Andygene AD8332 Hu, USA), PLTP (Andygene AD8193 Hu, USA), sPLA2 (Andygene AD12021 Hu, USA), LCAT (Andygene AD9164 Hu, USA), EL (Andygene AD8541 Hu, USA) levels in serum acording to manifacturer's instructions. Triacylglycerol levels were spectrophotometrically detected by Abbott Trigliserid (IL, ABD) kit. A chemiluminesence immunoassay analayzer (ADVIA Centaur XPT, Siemens Medical Solutions, USA) and a chemistry analyzer (Abbott Architect c16000, IL,ABD) were employed for cortisol and cholesterol analyses respectively.

5.7. Statistical methods : Normality of the quantitative data was assessed by Shapiro-Wilk test. Mean and standard deviation were used to summarize normally distributed data. The homogeneity of group variances were examined by Levene test. When group variances were homogeneous, one-way analysis of variance, otherwise Welch's test was used. For the variables that did not distributed normally or have a sample size less than 10, median, minimum and maximum values were used as descriptives. To perform group comparisons based on those variables, Kruskal-Wallis test and Conover pairwise comparison method were used. The distribution of the qualitative data were presented by count and percantage. Comparisons due to the those variables were performed by Pearson's chi-square test and Bonferroni correction was used for multiple comparisons. Two-sided significance level was accepted as 0.05 in all tests.

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