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A Possible Mechanism for Altered Immune Response in the Elderly

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Abstract. *Background: Reciprocal influences and bidirectional connections among the nervous, endocrine and immune systems, mediated by shared neuroendocrine hormones, chemo/cytokines and binding sites contribute to the maintainment of body homeostasis. The hypothalamus-pituitary axis may play an immunomodulating role and influence cellular immune responses by releasing various hormones and neuropeptides into the blood with direct modulatory action on the immune effectors, or by regulating the hormonal secretion of peripheral endocrine glands. Aging is associated with changes in immune function. The aim of this study was to evaluate circadian variations of some endocrine and immune factors in the elderly. Materials and Methods: Serum levels of cortisol, melatonin, thyrotropin-releasing hormone (TRH), thyroid stimulating hormone (TSH), free thyroxine (FT_4), growth hormone (GH), insulin-like growth factor (IGF) 1 and interleukin (IL) 2 were measured and lymphocyte subpopulation analyses were performed on blood samples collected every four hours for 24 hours from ten healthy young and middle-aged individuals (age 35-54 years) and from ten healthy elderly individuals (age 65-76 years). Results: There was a statistically significant difference between the groups in the observed values of CD20 and TSH serum levels (higher in the young and middle-aged) and CD25 and DR^+ T-cells (higher in the elderly). In the group of young and middle aged subjects, a clear circadian rhythm was validated for the time-qualified changes of all the factors studied, with the exception of FT_4, IGF1 and IL2. In the group of elderly individuals, a number of rhythms and correlations with neuroendocrine hormones were absent or altered. Conclusion: The results of the current study evidence aging-associated decrease of peripheral B-cell compartment, increase of activated T-cell compartment, decrease of hypophyseal thyrotropin secretion, altered circadian rhythmicity and altered hormone-immune cell correlations.*

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A number of age-related changes in the 24-hour hormonal and nonhormonal rhythms have been found in older human beings (1, 2). Numerous interactions exist among the nervous, endocrine and immune system, mediated by neurotransmitters, hormones and cytokines (3, 4). Lymphocyte subpopulations present circadian variation of some of their subsets and this variation may influence the magnitude and expression of the immune responses (5, 6). The immune response to active immunization tends to decrease and autoimmune phenomena tend to increase with aging (7). Immunologic response decreases in most elderly people and they produce much lower levels of antibody in response to vaccine than do younger people (9-11). The immune system must interact with neuro-endocrine structures and hormones to maintain body homeostasis and reciprocal influences among hypothalamus, pituitary, thyroid, adrenal, pineal gland and immune system have been shown (12). The pineal gland is innervated by post-ganglionic nervous fibers coming from the superior cervical ganglion, which receives fibers from the suprachiasmatic nucleus of hypotalamus, innervated by the retinohypothalamic tract. Changes in lighting condition influence activity of the retino-hypothalamic-pineal system, with inhibiting effect on melatonin production, controlled also by an endogenous free-running pacemaker located in the suprachiasmatic nucleus (13-19). Melatonin plays a role in immunomodulation in opiatergic ways and stimulates activated helper T lymphocytes to produce opioid agonists and cytokines (interleukin, IL2 and IL4). Opioid receptors were found in immunocompetent murine and human cells and these cells, activated by antigens or mitogens produce opioid peptides

and other substances (adrenocorticotropic hormone; thyroid stimulating hormone, TSH; growth hormone, GH; insulin like growth factor, IGF1; Vasoactive intestinal peptide). The immunomodulatory role of melatonin may also be exerted by an influence on the thymic function mediated by TRH and TSH, in experimental conditions able to counteract thymic involution induced by prednisolone (this effect seems to be thyroid-independent and not correlated to thyroxine levels). The influence of cortisol on immune function is well known and the total number of circulating lymphocytes has a nadir when adrenal hormone serum levels reach the zenith. GH and IGF1 have been recognized as stimulators of lymphopoiesis and immune function, an autocrine or paracrine GH/IGF1 system has been evidenced in lymphoid tissues, capable of influencing lymphopoiesis and immune function and in particular IGF1 assists the maturation of lymphocytes in bone marrow and their function in the periphery (20-22). We have evaluated the secretory profile of cortisol, melatonin, thyrotropin-releasing hormone (TRH), TSH, free thyroxine (FT_4), GH, IGF1 and IL2 and variations of lymphocyte subpopulations in a group of healthy elderly individuals, compared to a group of healthy young and middle-aged individuals, searching for differences that might explain aging-related changes of immune system function.

Materials and Methods

The participants gave written informed consent and the study was approved by the local Scientific and Ethical Committee. Peripheral blood samples were collected at intervals of four hours for 24 hours from ten healthy young and middle-aged individuals, aged 35-54 years (mean age±SEM 44.3±1.4 years, body mass index±SEM 25.3±1.2) and from ten healthy elderly individuals, aged 65-76 years (mean age±SEM 68.3±1.4 years, body mass index±SEM 25.7±1.5). Inclusion criteria were age (<65 years for the young and middle-aged, ≥65 and <80 years for the elderly), BMI (>25 and <30), no smoking status, normal physical activity level, no psychiatric disorder, no alcohol intake, no chronic conditions, normal blood pressure level. In all participants healthy status was assessed by medical history and physical examination, basal screening blood and urine test, ECG, chest X ray and upper and lower abdominal ultrasound scan. All partecipants were studied in our Department and were submitted to the same social routine (light/dark cycle and mealtimes). Sleep was allowed between 23:00 h (lights off) and 07:00 h (lights on). During daytime (between 07:15h and 20:15 h), partecipants stayed in the Department and standardized meals were provided at appropriate times for breakfast (07:30 h), lunch (12:30 h), and dinner (18:30 h).

For each blood sample, cortisol, melatonin, TRH, TSH, FT_4, GH, IGF1 and IL2 in serum and lymphocyte subpopulations (CD3, CD4, CD8, CD16, CD20, CD25, HLA-DR, TcRδ1) of peripheral blood anticoagulated with sodium ethylenediamine tetraacetic acid (EDTA) were measured. To measure hormone and IL2 serum concentrations, blood samples were centrifuged immediately after collection and frozen at –20˚C for later determination. All samples were analyzed in duplicate in a single assay; the intrassay and interassay coefficients of variation were below 10% and 9% respectively for cortisol, 13% and 16% for melatonin, 5% and 6% for TRH, 8% and 7% for TSH, 4% and 6% for FT_4, 5% and 3% for GH, 3% and 8% for IGF I, 5% and 7% for IL-2.

Cortisol was measured by polarized light immuno-fluorescence assay (Cortisol TDx/TDxFLx; Abbott Laboratories, Abbott Park, IL, USA), melatonin by radioimmunoassay (Melatonin Radioimmunoassay Kit, Nichols Institute Diagnostics, San Clemente, CA, USA), TRH by radioimmunoassay (Frederic Joliot-Curie National Research Institute for Radiobiology and Radiohygiene, Budapest, Hungary), TSH by immunoenzymatic assay (Enzymun-Test TSH; Boehringer Mannheim Immunodiagnostics, Mannheim, Germany), FT_4 by immunoenzymatic assay (Enzymun-Test FT_4;Boehringer Mannheim Immunodiagnostics, Mannheim, Germany), GH by immunoenzymometric assay (AIA-PACK HGH, Tosoh, Japan), IGF1 by radioisotopic assay (IGF I 100T Kit, Nichols Institute Diagnostics, San Clemente, CA, USA) and IL-2 by immunoenzymatic assay (IL-2 EIA,Technogenetics, Sesto San Giovanni, Milano, Italy).

Analyses of lymphocyte subpopulations were performed on unfixed cell preparations with a multicolor fluorescence activated cell sorter (FACScan; Becton-Dickinson FACS Systems, Sunnyvale, CA, USA) and a panel of monoclonal antibodies (mAbs) to lymphocyte surface antigens (OKT3, OKT4, OKT8, OK-NK, OKB20, OKT26a, OK-DR Ortho Diagnostic Systems; TcRδ1 Medical Systems). Briefly, mAbs were directly conjugated with phycoerythrin (PE), peridin chlorophyll protein (PerCP), allophycocyanine (APC) and fluorescein isothiocyanate (FITC). Ten microliters of mAbs were added to 100 ml EDTA blood in Trucount tubes (BD Biosciences, San Jose, CA, USA). After a 15-min incubation at room temperature, the erythrocytes were disintegrated and after centrifugation the supernatants were washed with PBS. Non-lymphocytic cells contaminating the preparations were excluded from analysis using scatter gates set on the 90˚ light scatter profile. At least 10000 cells were acquired on the FACScan. Absolute counts of T-cell subsets were calculated based on the proportion of the respective T cell subpopulation and on absolute counts obtained by the procedure. The number of fluorescent cells was expressed as a percentage of the total lymphocytes.

Statistical analysis. Statistical evaluation of hormone serum levels and of percentages of cells was performed by non inferential descriptive biometric analysis (Student's *t*-test and Mann-Whitney rank sum test, as indicated, to compare areas under the curve (AUCs), calculated according to the trapezoidal method and Pearson's product moment correlation coefficients calculated for hormone serum levels and percentages of cells at each sampling time to assess temporal relationships between variations in lymphocyte subpopulations and variations in hormone concentrations). Inferential temporal descriptive biometric analysis were carried out using the methods named Single Cosinor and Population Mean Cosinor, based on a least square fit of a cosine wave to individual and group time series data, testing the occurrence (whether the zero-amplitude assumption is rejected at a probability level $p<0.05$) and quantifing the parameters MESOR, Amplitude and Acrophase of consistent pattern of circadian rhythm. MESOR is the acronym for midline estimating statistic of rhythm and defines the rhythm-determined average. Amplitude is the measure of one half the extent of rhythmic change in a cycle estimated by the function used to approximate the rhythm. Acrophase, a measure of timing, is the phase angle of the crest time in the function

appropriately approximating a rhythm, in relation to the specified reference timepoint. Rhythms with a frequency of 1 cycle per 20±4 h are designated circadian, rhythms with a frequency higher than 1 cycle per 24 h are designated as ultradian, rhythms with a frequency lower than 1 cycle per 24 h are designated as infradian (8).

Results

Table I shows integrated time-qualified 24-hour percentage values and hormone serum levels expressed as area under the curve (AUC)±s.e. There was a statistically significant difference between the groups in the observed values of CD20 (total B-cells, higher in the young and middle aged, p=0.01), CD25 (activated T-cells with expression of the α chain of interleukin 2 receptor, higher in elderly subjects, p=0.003), DR^+ T-cells (activated T lymphocytes, higher in the elderly, p=0.02) and TSH serum levels (higher in the young and middle aged, p=0.02). There was no statistically significant difference in the observed values of CD3 (total T lymphocytes), CD4 (helper/inducer T-cells), CD8 (suppressor/cytotoxic T-cells), CD4/CD8 ratio, CD16 (natural killer cells), HLA-DR (B-cells and activated T-cells), TcRδ1 (epitope of the constant domain of δ chain of T-cell receptor1), cortisol, melatonin, TRH, FT_4, GH, IGF1 and IL2.

Table II shows chronobiological data derived from best fitting sine curves (fitted period: 24 hours = 360°). In the group of young and middle aged individuals a clear circadian rhythm was validated for the time-qualified changes of all the factors studied except for FT_4, IGF1 and IL2. In the group of elderly individuals a clear circadian rhythm was validated for the nyctohemeral changes of CD3 (with a phase delay of 3 hours), CD8, CD4/CD8 ratio, CD16, cortisol (with a phase delay of 1 hour), melatonin and TSH (with a phase delay of 1 hour).

Figure 1 shows 24-hour profile of lymphocyte subpopulations in young and middle aged individuals. Figure 2 shows 24-hour profile of lymphocyte subpopulations in elderly individuals. Figure 3 shows 24-hour profile of cortisol, melatonin, TRH and TSH serum levels in young and middle aged individuals and in elderly individuals. Figure 4 shows 24-hour profile of FT_4, IL2, GH and IGF1 serum levels in young and middle aged individuals and in elderly individuals. Figure 5 shows correlations at 06:00h among hormone serum levels and lymphocyte subset percentages in young and middle aged individuals and in elderly individuals. Figure 6 shows correlations at 22:00h among hormone serum levels and lymphocyte subset percentages in young and middle aged individuals and in elderly individuals.

Pearson product moment correlations showed that in young and middle aged individuals at 06:00h, CD3 correlated positively with TRH (r=0.78, p=0.007) and FT_4 (r=0.67, p=0.03), CD4 correlated negatively with TRH (r=–0.64, p=0.04) and IL2 (r=–0.59, p=0.05), CD8 correlated positively with TRH (r=0.80, p=0.005) and with FT_4 (r=0.74, p=0.01), CD16 correlated positively with TRH (r=0.84, p=0.002), with TSH (r=0.74, p=0.01) and with FT_4 (r=0.72 p=0.01); CD25 correlated positively with TRH (r=0.62, p=0.05) and with FT_4 (r=0.66, p=0.03); TCRγδ correlated positively with TSH (r=0.69, p=0.02). At 22:00h, CD3 correlated positively with FT_4 (r=0.73, p=0.01) and with IGF1 (r=0.74, p=0.01); CD4 correlated negatively with IGF1 (r=–0.73, p=0.01), CD8 correlated negatively with melatonin (r=–0.60, p=0.05) and positively with IGF1 (r=0.97, p<0.001), CD16 correlated positively with IGF1 (r=0.60, p=0.05); CD25 correlated positively with TRH (r=0.86, p=0.001), with FT_4 (r=0.83, p=0.002) and with IL2 (r=0.60, p=0.05).

Pearson product moment correlations showed that in the elderly at 06:00h, CD8 correlated negatively with melatonin (r=–0.63, p=0.04), CD16 correlated negatively with FT_4 (r=–0.69, p=0.02); CD20 correlated negatively with FT_4 (r=–0.76, p=0.009); CD25 correlated negatively with FT_4 (r=–0.64, p=0.04); TCRγδ correlated negatively with GH (r=–0.80, p=0.004) and positively with IL2 (r=0.77, p=0.008). At 22:00h CD4 correlated positively with FT_4 (r=0.66, p=0.03) and with IL2 (r=0.65, p=0.04); CD8 correlated negatively with IL2 (r=–0.67, p=0.03), CD16 correlated positively with IGF1 (r=0.60, p=0.05), CD25 correlated negatively with TRH (r=–0.89, p<0.001) and positively with IGF1 (r=0.80, p=0.004), TCRγδ correlated negatively with GH (r=–0.69, p=0.02).

Table I. *Integrated time-qualified 24-hour values expressed as area under curve (AUC)±SE.*

Parameter	Young and middle aged	Elderly
CD3	1587.68±44.24	1647.03±27.32
CD4	878.33±63.53	862.71±32.41
CD8	605.75±93.12	612.62±32.35
CD4/CD8	34.74±10.47	28.82±1.85
CD16	142.52±22.31	172.73±32.35
CD20	265.45±31.86	170.02±20.62*
CD25	74.12±13.96	146.9±26.63*
DR+T–cells	61.3±1.71	113.5±8.18*
HLA-DR	318.03±24.31	302.82±20.71
TcRδ1	61.71±11.70	83.13±9.33
Cortisol	243.83±13.31	303.17±33.53
Melatonin	584.04±38.42	560.55±33.31
TRH	9.23±1.25	8.27±1.62
TSH	32.66±4.08	23.42±2.65*
FT_4	23.86±1.42	24.01±1.19
GH	4.12±0.23	7.65±1.26
IGF1	4621.22±223.72	4321.65±498.37
IL2	7.41±0.46	9.35±1.31

All parameters analyzed in all the subjects. Units: % for lymphocyte subpopulations, µg/dl for cortisol, pg/ml for melatonin, ng/ml for TRH, µU/ml for TSH, ng/dl for FT4, ng/ml for GH, ng/ml for IGF1, IU/ml for IL2; *p<0.05.

Table II. *Chronobiological data derived from best fitting sine curves (fitted period: 24 hours=360°).*

	Young and middle-aged individuals				
Factor	*p*-Value	MESOR±SE	Amplitude±SE	Acrophase±SE (°)	Time (H:Min±SE)
CD3	0.002	78.04±0.20	1.14±0.28	23.4±14.3	01:34±00:57
CD4	0.001	44.98±0.83	3.13±1.17	5.2±16.1	00:21±01:04
CD8	0.002	28.99±0.11	1.68±0.22	177±8.3	11:48±00:32
CD4/CD8	0.001	1.54±0.04	0.21±0.3	6.3±0.2	00:25±00:01
CD16	0.003	6.43±0.45	0.85±0.75	215.6±46.6	14:22±03:06
CD20	0.013	12.87±0.27	1.70±0.32	342.2±14.3	22:49±00:57
CD25	0.002	3.35±0.03	0.39±0.11	6.3±4.2	00:25±00:17
DR+ T-cells	0.005	3.33±0.40	0.46±0.90	0.42±52.2	00:02±03:29
HLA-DR	0.012	13.73±0.23	1.73±0.34	334.6±14.5	22:18±00:58
TcRδ1	0.003	2.86±0.13	0.28±0.14	137.4±12.3	09:10±00:49
Cortisol	0.002	11.04±1.43	6.43±2.21	123.3±21.6	08:13±01:26
Melatonin	0.005	37.99±4.22	26.23±6.34	23.3±11.1	01:33±00:44
TRH	0.022	0.43±0.03	0.03±0.02	52.1±12.3	03:28±00:49
TSH	0.002	1.62±0.02	0.47±0.07	334.0±14.7	22:16±00:59
FT_4	0.931	1.25±0.05	0.01±0.02	15.5±213.3	01:02±14:13
GH	0.001	0.38±0.88	0.34±0.15	13.7±17.2	00:55±01:09
IGF-I	0.437	223.43±1.23	17.43±1.73	122.6±4.4	08:10±00:18
IL-2	0.426	0.38±0.08	0.01±0.02	314.5±35.6	20:58±02:22
	Elderly individuals				
Factor	*p*-Value	MESOR±SE	Amplitude±SE	Acrophase±SE (°)	Time (H:Min±SE)
CD3	0.002	82.45±0.05	1.02±0.02	72.2±3.0	04:49±00:12
CD4	0.136	46.22±0.90	3.23±1.42	32.3±21.3	02:09±01:25
CD8	0.008	28.95±0.42	3.22±0.30	184.7±12.2	12:19±00:49
CD4/CD8	0.009	1.30±0.03	0.24±0.6	16.7±16.0	01:07±01:04
CD16	0.002	8.76±0.23	2.51±0.76	194.3±9.3	12:57±00:37
CD20	0.285	8.60±0.38	1.12±0.31	236.4±45.3	15:46±03:01
CD25	0.151	7.34±0.46	1.04±0.23	253.4±11.1	16:54±00:44
DR+ T-cells	0.344	5.11±0.21	1.14±0.3	133±13.6	08:52±00:54
HLA-DR	0.294	14.44±0.32	1.21±0.1	178.9±39.1	11:56±02:36
TcRδ1	0.263	4.32±0.12	0.32±0.12	186.9±35.4	12:28±02:22
Cortisol	0.016	13.26±0.60	5.63±1.03	133.6±12.5	08:54±00:50
Melatonin	0.001	46.74±6.51	37.13±5.27	13.4±13.5	00:54±00:54
TRH	0.544	0.45±0.03	0.03±0.01	0.1±51.1	00:00±03:24
TSH	0.004	1.07±0.02	0.39±0.03	355.0±5.4	23:40±00:22
FT_4	0.488	1.23±0.31	0.01±0.11	211.0±34.3	14:04±02:17
GH	0.001	0.44±0.04	0.34±0.02	354.3±3.4	23:37±00:14
IGF-I	0.680	214.34±3.24	2.33±4.21	172.4±122.8	11:30±08:11
IL-2	0.462	0.43±0.03	0.02±0.04	215.5±35.0	14:22±02:20

Units: % for lymphocyte subpopulations, μg/dl for cortisol, pg/ml for melatonin, ng/ml for TRH, μU/ml for TSH, ng/dl for FT_4, ng/ml for GH, ng/ml for IGF-I, IU/ml for IL-2; all parameters analyzed in all the subjects; *p*-value from an F-test of the null amplitude rejection hypothesis (for a rhythm with a chosen period τ).

Discussion

The contribution of the immune system to healthy aging and longevity is still an open question and immunosenescence is a process that affects all cell compartments of the immune system (23-27). The results obtained in our study show interesting differences between the studied groups in hematic levels and temporal organization of some investigated factors. Young and middle aged individuals have higher levels of total B-cells and show a clear circadian rhythm and a proper temporal architecture of many studied factors. The T suppressor/cytotoxic lymphocytes, natural killer cells and the levels of TcRδ1 are higher in the late morning/at noon and show a clear circadian rhythmicity, suggesting that T-cell receptor (TCR)γδ complex is mainly expressed at the cell surface of cellular elements temporally and may be

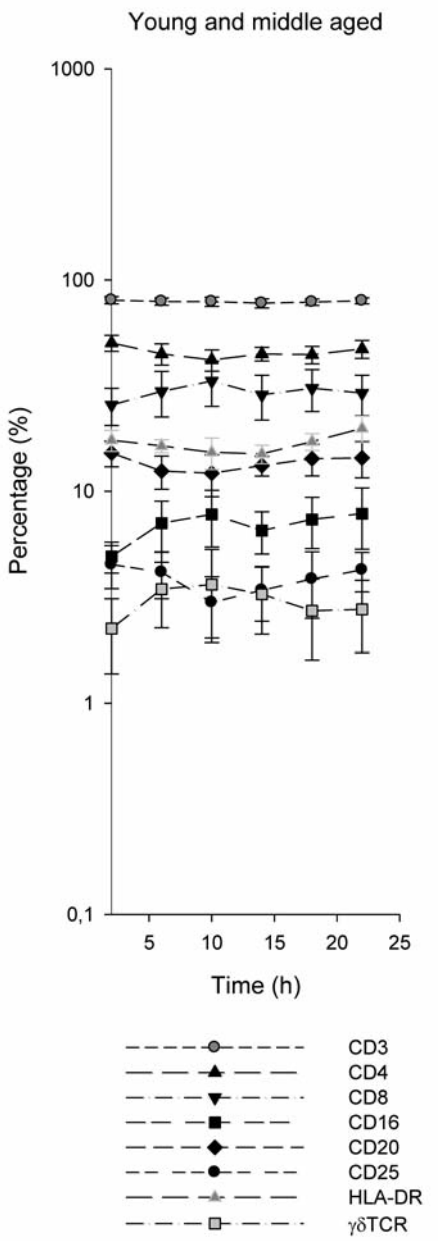

Figure 1. *Plot showing 24-hour time qualified profiles of lymphocyte subset percentages expressed on a common logarithmic scale as mean±SE calculated on single time point values from ten young and middle aged individuals. Percentages of circulating CD3, CD4, CD20, CD25 and HLA-DR bearing cells show circadian rhythmicity with acrophase during the night. Percentages of circulating CD8, CD16 and γδTCR bearing cells show circadian rhythmicity with acrophase during the day.*

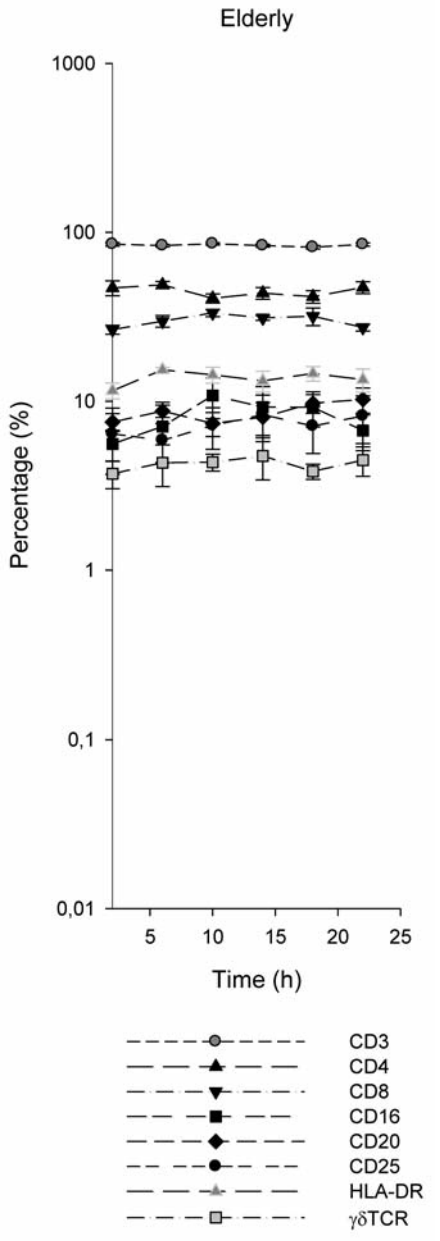

Figure 2. *Plot showing 24-hour time qualified profiles of lymphocyte subset percentages expressed on a common logarithmic scale as mean±SE calculated on single time point values from ten elderly individuals. Percentages of circulating CD3 bearing cells show circadian rhythmicity with acrophase during the night. Percentages of circulating CD8 and CD16 bearing cells show circadian rhythmicity with acrophase during the day. Percentages of circulating CD4, CD20, CD25, HLA-DR and γδTCR bearing cells do not show circadian rhythmicity.*

functionally related to NK and cytotoxic T lymphocytes. Previous studies have shown that this complex is involved in T-cell activation and that activated TCRγδ-expressing cells frequently exhibit cytotoxic activity against multiple target cell lines including neoplastic cells. There is some speculation that TCRγδ-expressing cells may be specialized for mycobacterial immunity or destruction of 'stressed' autologous cells which show increased expression of heat-shock proteins and the

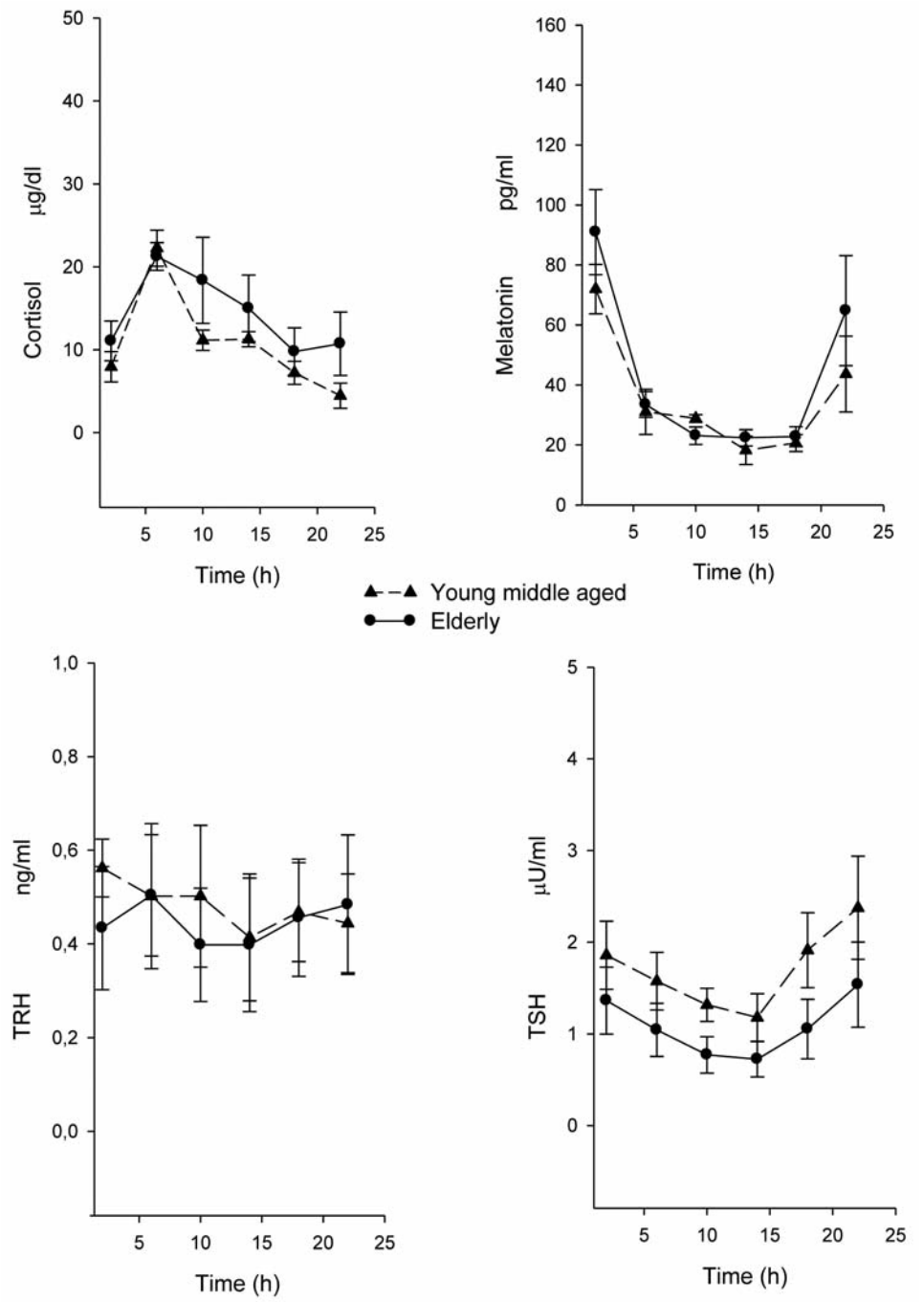

Figure 3. *x-y plot showing 24-hour time qualified profiles of cortisol, melatonin, TRH and TSH serum levels expressed as mean±SE calculated on single time point values from ten young and middle aged individuals and ten elderly individuals. A clear circadian rhythm is validated for the time-qualified changes of all the factors studied except for TRH serum level variations in the elderly.*

presence of TCRγδ-expressing cells exhibiting *in vitro* lymphokine activated killer activity against autologous acute leukemia cells has recently been demonstrated (28-31). As evidenced in our study, peripheral blood lymphocytes show circadian variations of specific subpopulations and the T helper/inducer and the T suppressor/cytotoxic subsets change with circadian rhythmicity but in opposing phases, showing a temporal organization of lymphocyte functions. The variations of total T-cells, T helper/inducer subset, DR+ B-cells and activated T-cells, total B-cells and activated T-cells with

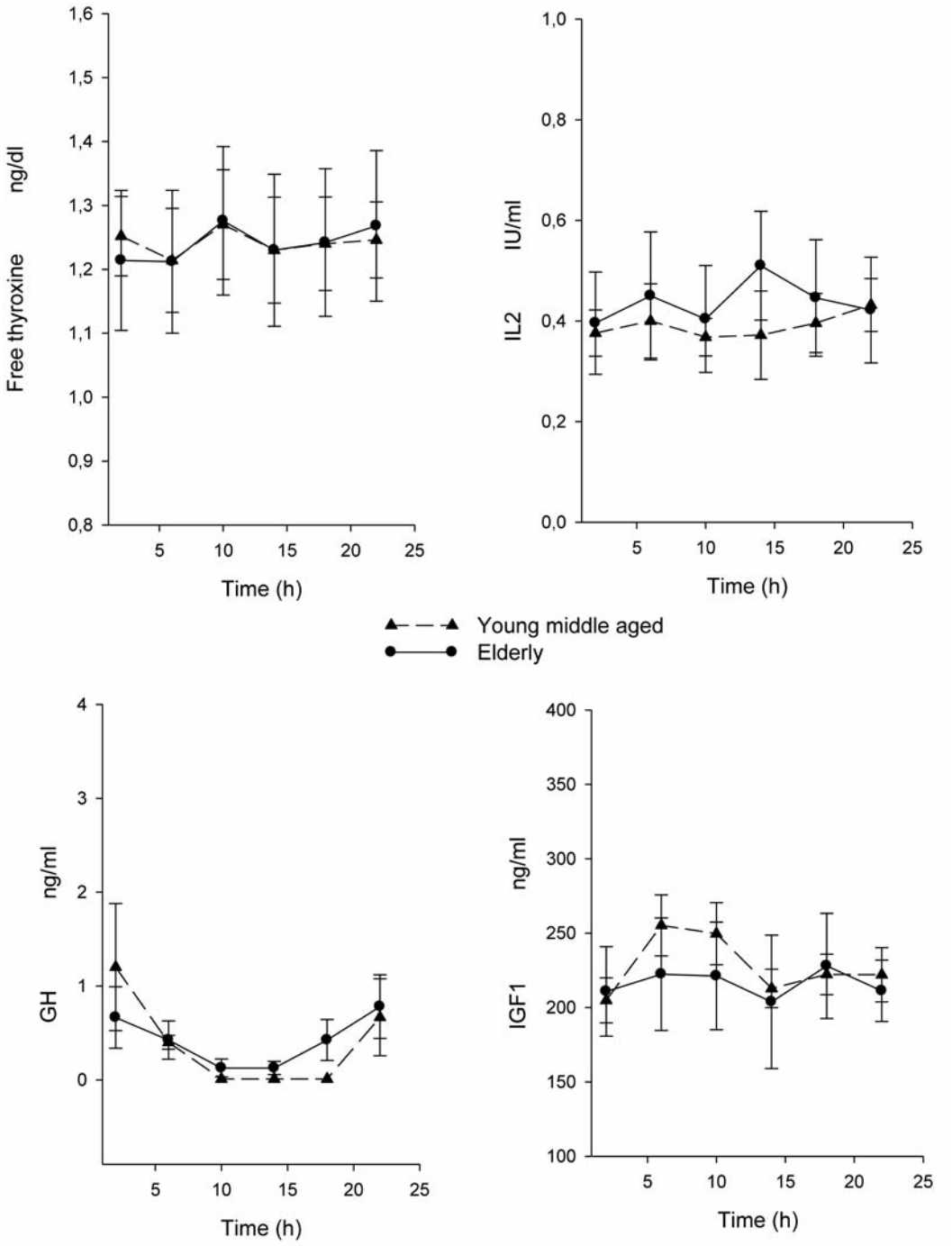

Figure 4. *Plot showing 24-hour time qualified profiles of FT_4, IL2, GH and IGF1 serum levels expressed as mean±SE calculated on single time point values from ten young and middle aged individuals and ten elderly individuals. A clear circadian rhythm is validated for the time-qualified changes of GH serum level variations in the young and middle aged individuals and in the elderly.*

expression of the α chain of IL2 receptor are synchronized with those of melatonin, TRH, TSH, and GH, in antiphase with the rhythm of cortisol.

The elderly in our study have higher levels of DR^+ and $CD25^+$ T lymphocytes and we have evidenced that in these individuals the circadian rhythm of total T-cells, cortisol and TSH is phase delayed, whereas the nyctohemeral variations of T helper/inducer subset, DR^+ B-cells and activated T-cells, total B-cells, $CD25^+$ cells, DR^+ T-cells, TCRγδ-expressing cells and TRH serum levels do not show circadian periodicity.

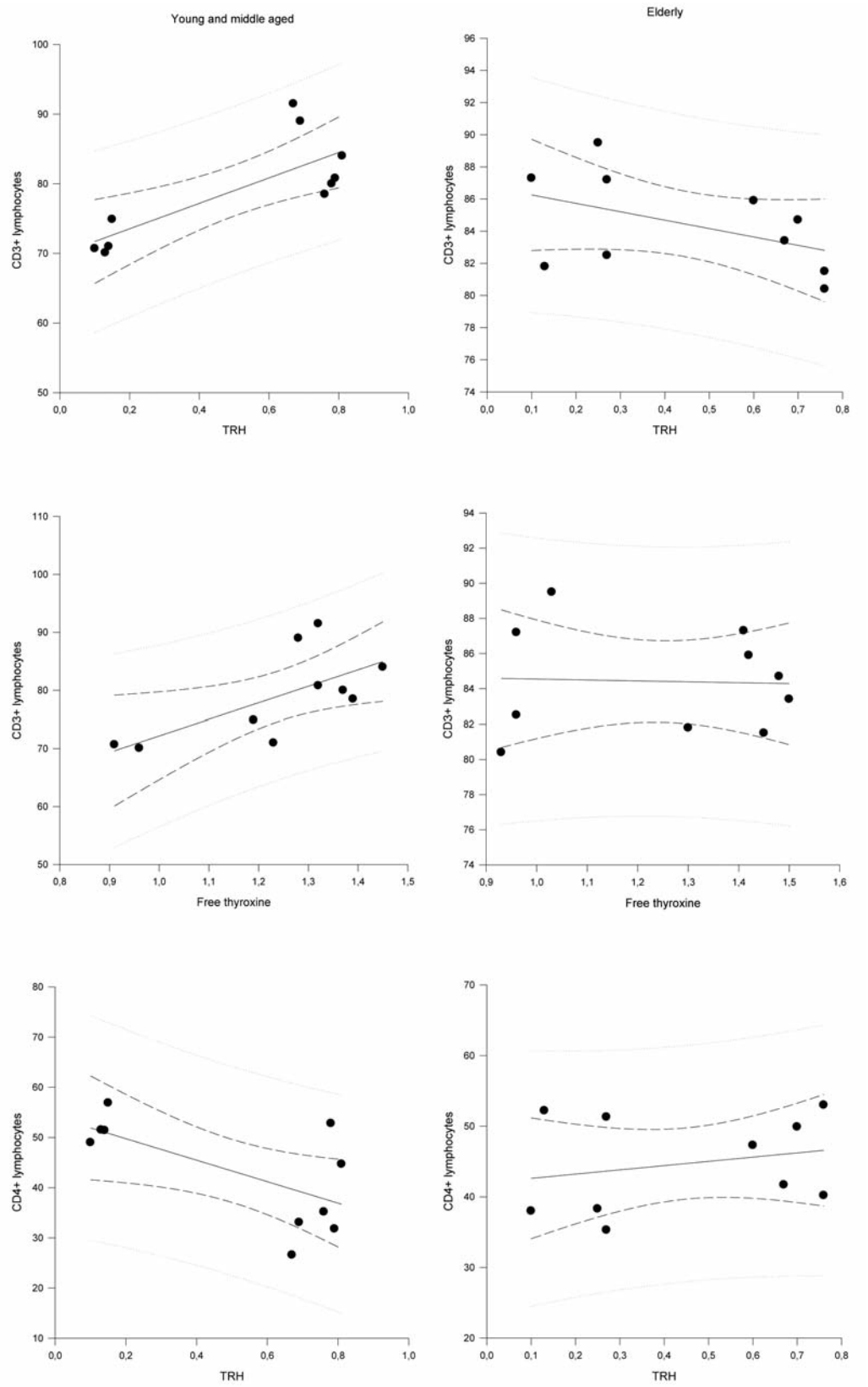

Figure 5. *Correlations at 06:00h among hormone serum levels and lymphocyte subset percentages in young and middle aged individuals and in the elderly. (Continued)*

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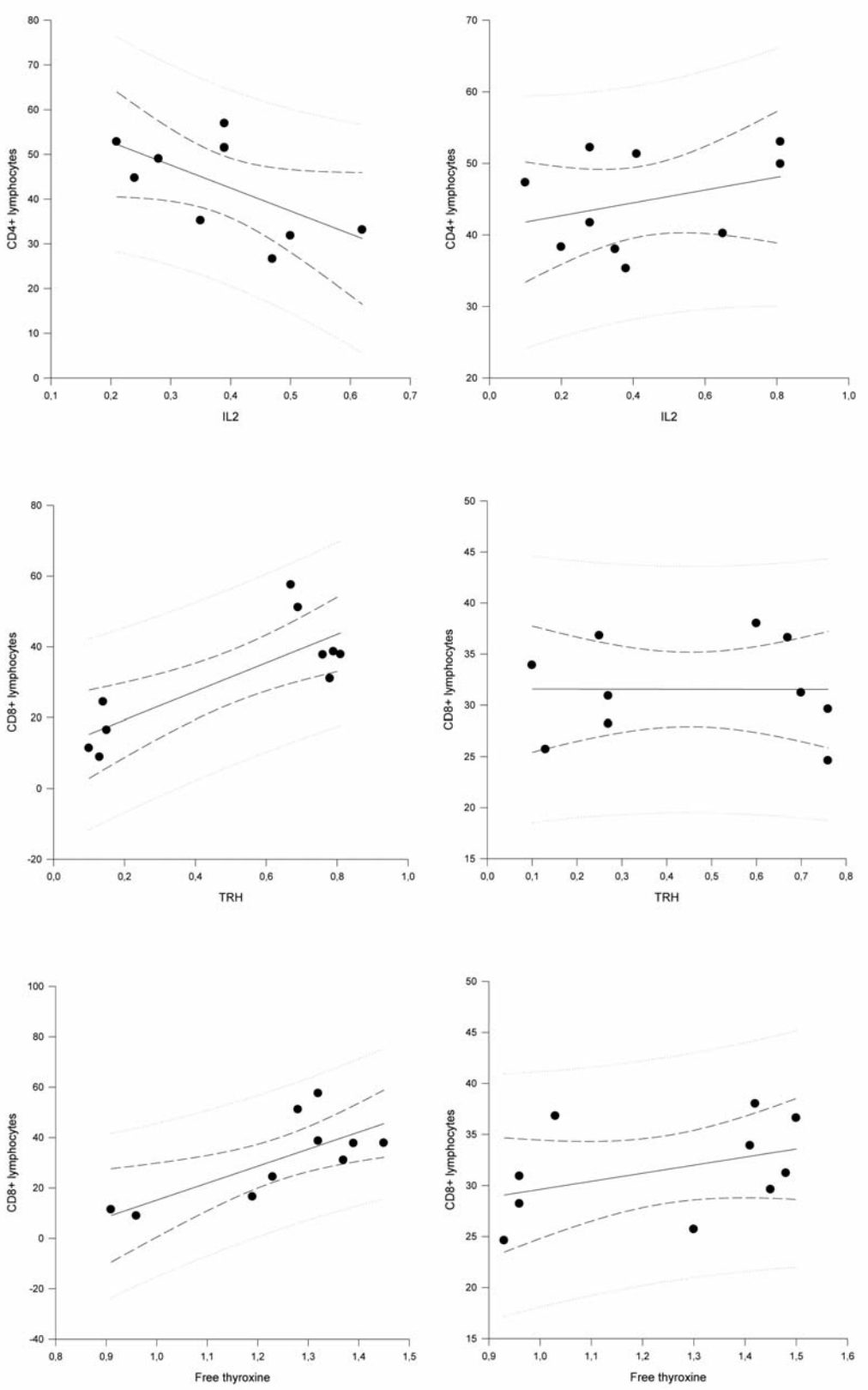

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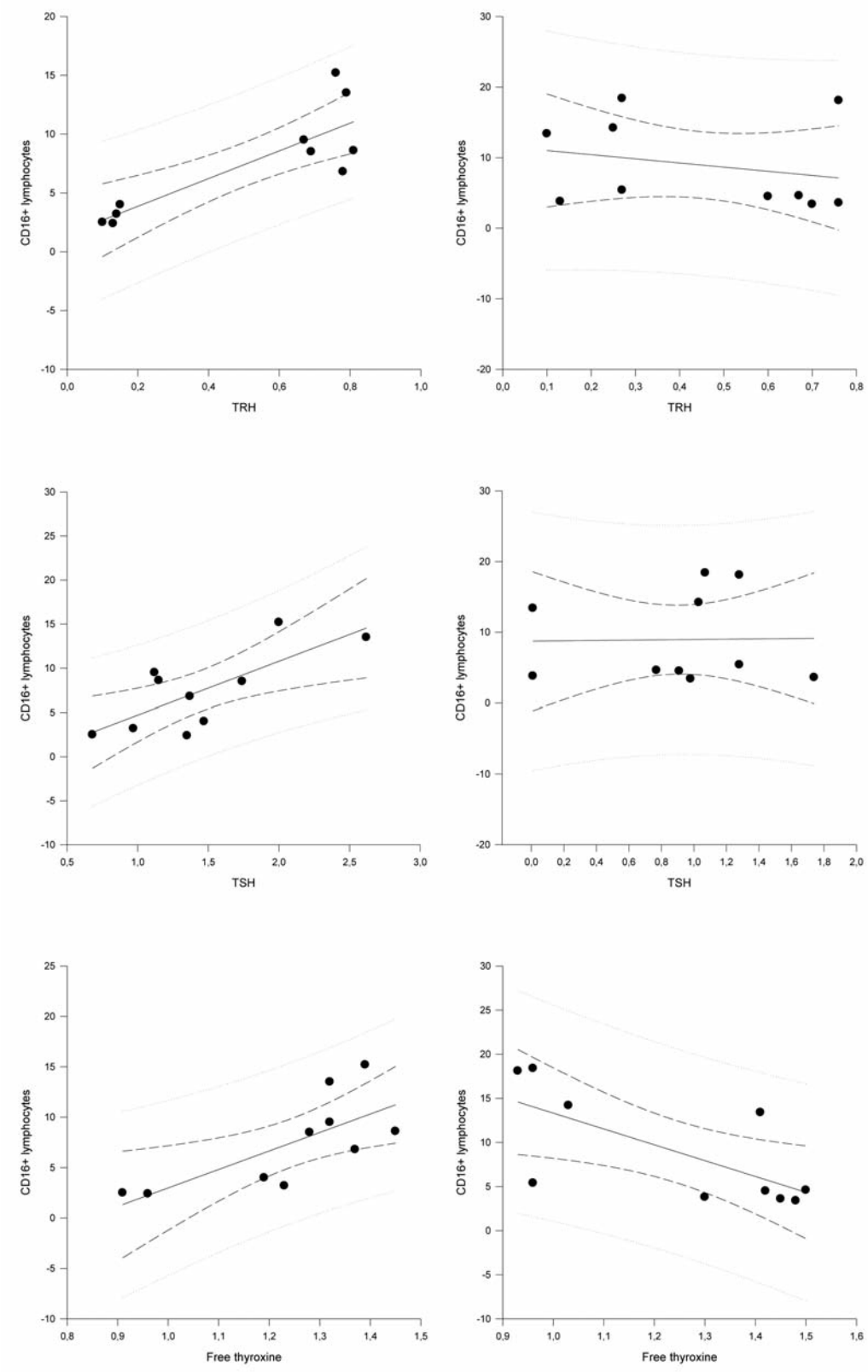
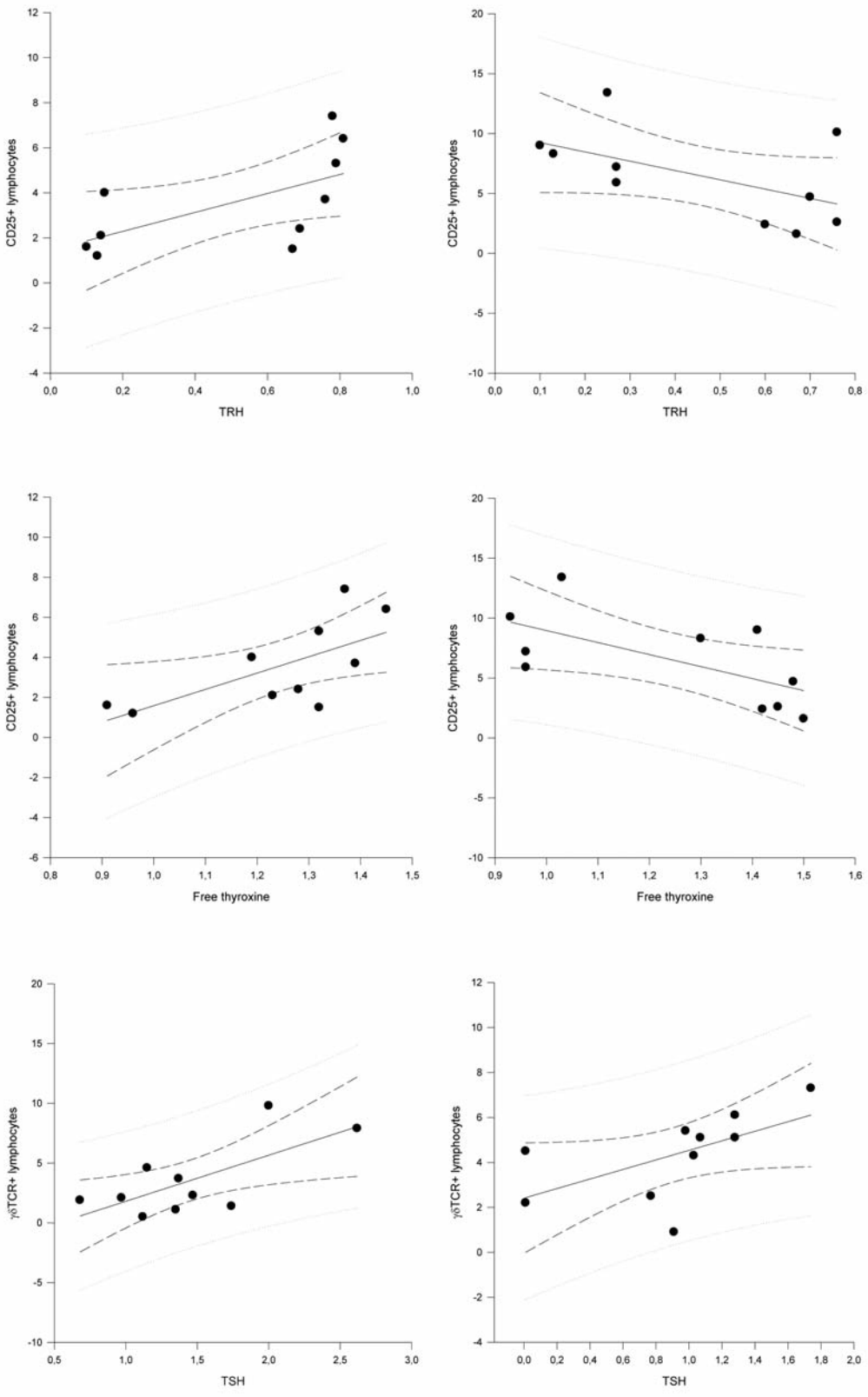


Figure 5. *continued*

Figure 5. *continued*



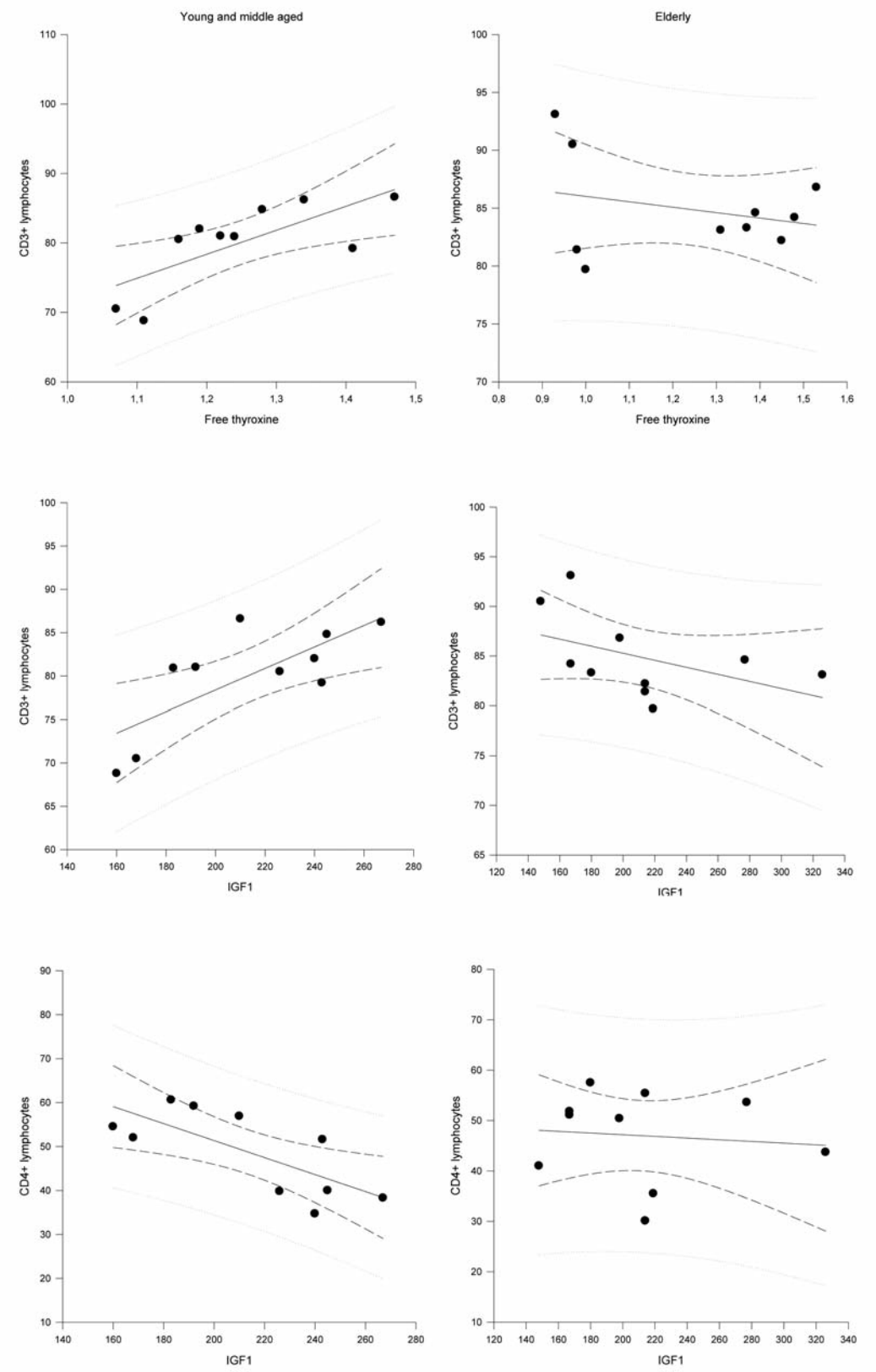


Figure 6. *Correlations at 22:00h among hormone serum levels and lymphocyte subset percentages in young and middle aged individuals and in the elderly. (Continued)*

Figure 6. *continued*

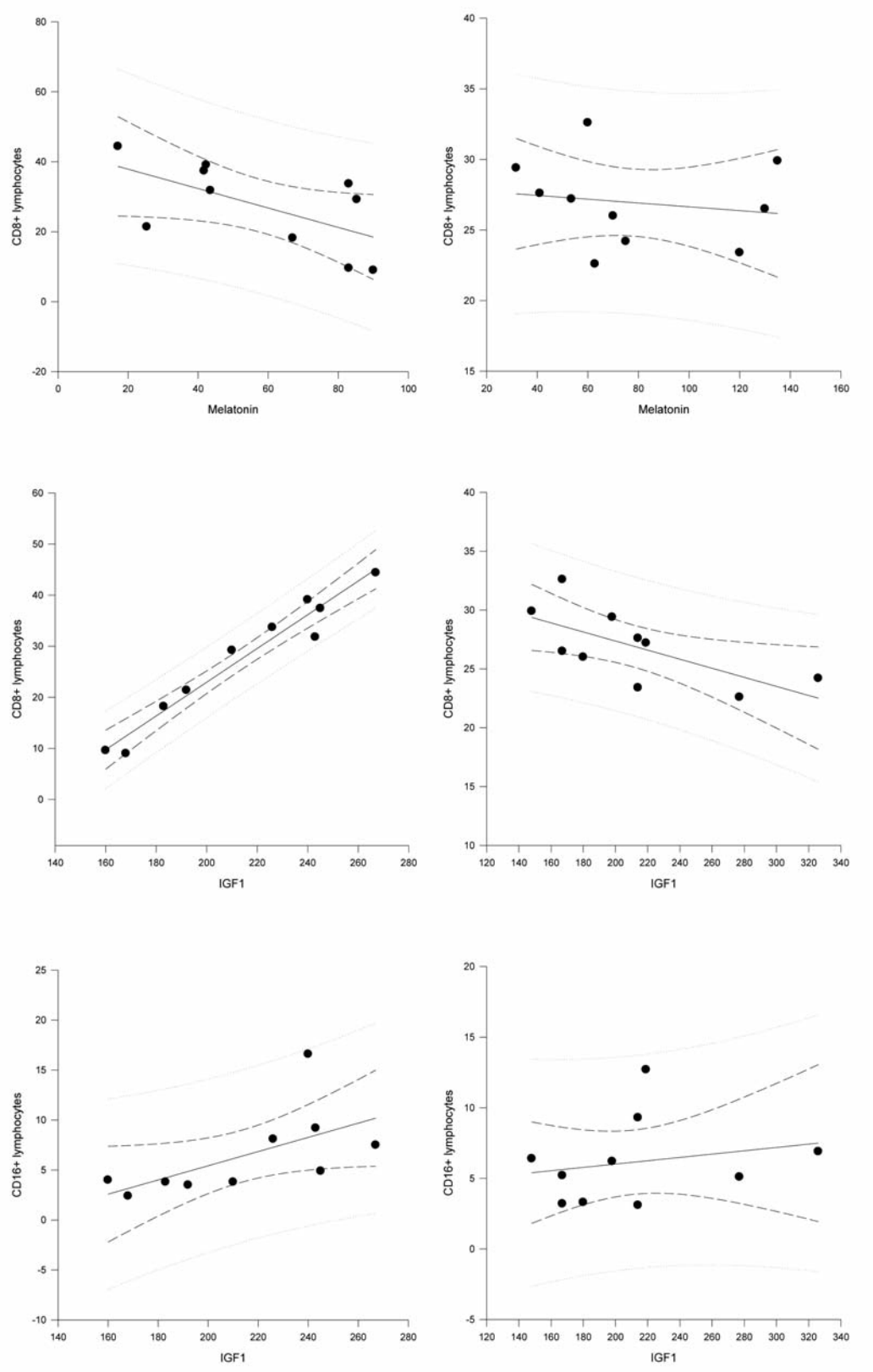
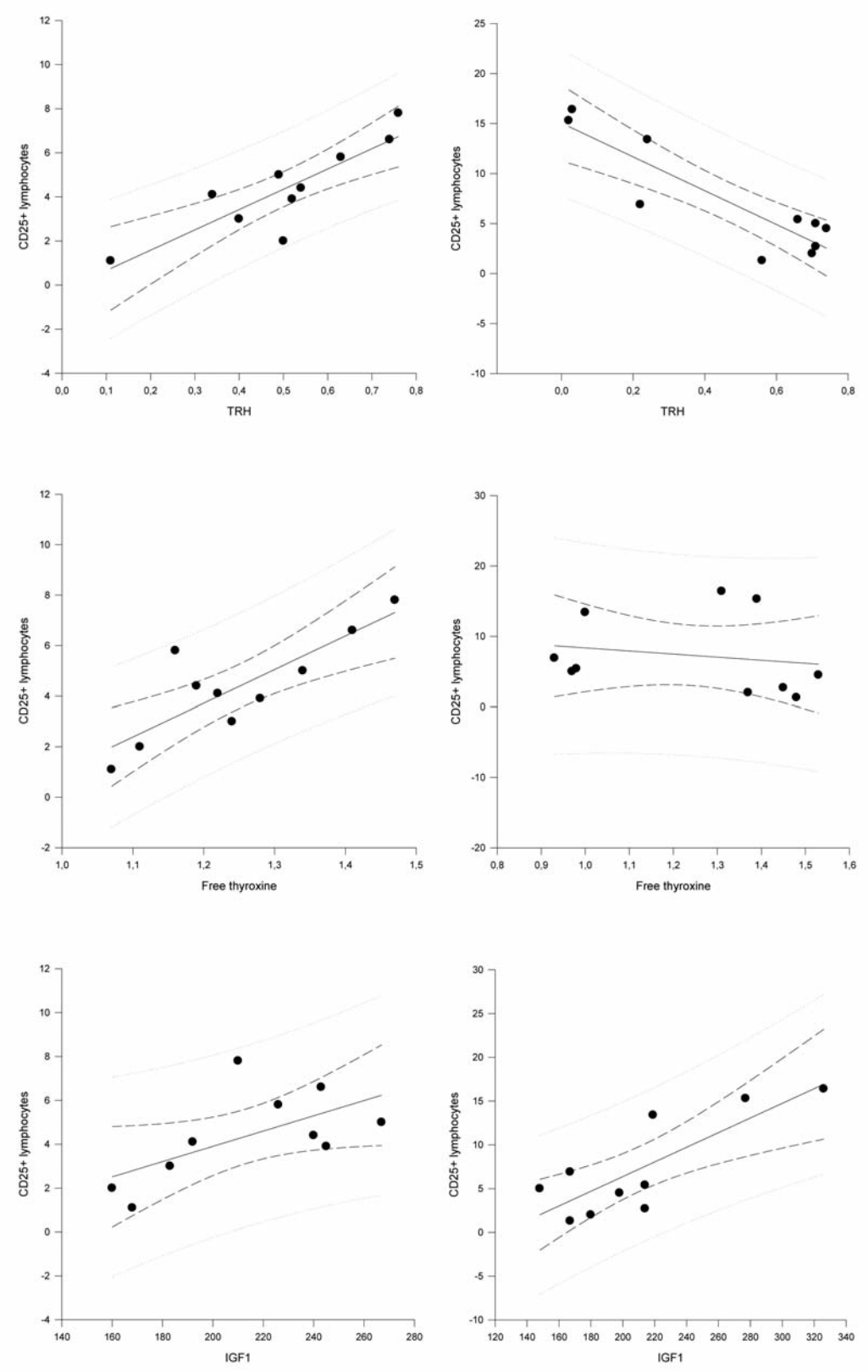


Figure 6. *continued*

Figure 6. *continued*



Alteration of circadian rhythmicity may be responsible for altered correlations among lymphocyte subpopulation and hormone time-related variations, which may cause loss of physiological timed windows of interaction with occurrence of new anomalous interactions. In our study, we found a number of positive correlations among lymphocyte subsets and TRH, TSH, free thyroxine and IGF1, among others, in the young and middle aged compared to the elderly. TSH has been shown to have a variety of immune regulating cytokine-like activities that can affect the magnitude of antibody and cell-mediated responses of peripheral lymphocytes. TRH and TSH enhance lymphocyte activity, but the major concern is to know whether their effects on immune responses are direct or are related to their regulation of the secretion of thyroid hormones. Enhanced lymphoid responses, achieved with high levels of triiodothyronine and thyroxine, but low levels of TSH and TRH – and with the converse in hypothyroid conditions – strengthen the possibility that levels of thyroid hormones modulate lymphocyte reactivity independently of TSH and TRH levels. Thyroid hormones play critical roles in differentiation, growth and metabolism, but their participation in immune system regulation has not been completely elucidated. A recent study evidences the important role of thyroid hormones in regulating lymphocyte reactivity *via* the regulation of protein kinase C content in lymphocytes, which could be involved in altered responsiveness to mitogen-induced stimulation of proliferative responses (32).

In our study, the elderly have lower TSH serum levels and do not show circadian rhythmicity of a number of lymphocyte subpopulations and of TRH variation, this phenomenon might explain the loss of correlation or the negative correlation evidenced among the hypothalamic protirelin/hypophyseal thyrotropin and key lymphocyte subsets (CD3, CD8, CD16, CD25 and TCRγδ-expressing cells). Hormone alterations are more evident in the very elderly (age>80 years) and this group needs to be studied separately; this is why we have not considered them in our study (33).

Besides the immunomodulatory action of hypothalamus-pituitary-thyroid axis hormones, GH and IGF1 have been demonstrated to promote hematopoiesis, particularly the megakaryocyte and erythroid lineages, both *in vitro* and *in vivo*, and to promote early B-cell and natural killer cell development in the bone marrow inducing B-cell proliferation and immunoglobulin (Ig) production and promoting the survival of T-cell progenitors and their development in the thymus. GH and IGF1 have been found to promote T-cell chemotaxis and therefore may play a role in normal lymphocyte circulation to the lymphnodes and spleen.

Data obtained in our study show loss of correlation among some lymphocyte subsets (CD3- and CD8-bearing cells) and IGF1 and the presence of positive correlation among IGF1 and CD16- and CD25-bearing cells. Total IGF1 and IL2 serum levels do not show circadian rhythmicity. In contrast, lymphocyte subset percentages change following a specific pattern and the severe alteration of nyctohemeral variation found in the changes of DR- and CD25-expressing lymphocytes in the peripheral blood of the elderly might cause an anomalous time-related interaction with immunomodulatory hormones, cytokine and chemokines and anomalous functioning of activated T lymphocytes. Increased classical signs of T-cell activity is the increase of soluble interleukin 2 receptor in serum and up-regulation of HLA-DR and interleukin 2 receptor on circulating T lymphocytes. IL2 plays a pivotal role in regulating the adaptive immune system by controlling the survival and proliferation of regulatory T-cells, which are required for the maintenance of immune tolerance. Moreover, IL2 is implicated in the differentiation and homeostasis of effector T-cell subsets, including T helper1, T helper2, T helper17, and memory $CD8^+$ T-cells. The IL2 receptor is composed of 3 distinct subunits, namely the alpha (CD25), beta (CD122), and gamma (gammac) chains. Of crucial importance for the delivery of IL-2 signals to regulatory T-cells is the expression of CD25, which, along with CD122 and gammac, confers high affinity binding to IL2. Notably, recent findings suggest a novel role for CD25, whereby CD25 molecules on regulatory T-cells and possibly other cells are capable of influencing T-cell homeostasis by means of IL2 deprivation (34, 35).

Our data evidence important alterations in some parameters of the immune system during aging. The decrease of B-cells, lymphocytes that play a key role in the humoral immune response, may be responsible for a decreased response to exogenous antigens, included vaccines and adjuvants. In most elderly people, response is sufficient to confer protection, but they need to be revaccinated with some vaccines or toxoids because of waning response. In the same way, there is a need for influenza vaccines with improved efficacy in the elderly. This need is underscored by both the observation that influenza has a major clinical and economic impact in the elderly and the fact that currently available vaccines are generally less effective in the elderly than in younger individuals (36-39). Approaches currently available to meet this medical need in older adults may include the use of adjuvanted vaccines and future strategies under evaluation include the use of high-dose vaccines, novel or enhanced adjuvantation of current vaccines, use of live attenuated vaccines in combination with current vaccines, DNA vaccines, recombinant vaccines as well as the use of alternative antigens. A novel antigen-presenting strategy to overcome impaired immune responses is the use of virosome vaccine delivery system and the use of different modes of delivery (intradermal or intranasal) (40, 41). On the other hand, the increase of activated T-cells may be associated with an increased frequency of autoimmune phenomena and an altered regulation of immune function.

In our study, we have shown that the circadian rhythmicity of these subsets is severely altered in the elderly. In a recent study (42), the circadian variation of lymphocyte subsets was related to cortisol and catecholamine (epinephrine) influence on cell redistribution to the bone marrow, mobilization and migration to lymphoid and non lymphoid organs and peripheral tissues. As evidenced in our study, there are statistically significant time-qualified correlations among lymphocyte subset percentages and hormone serum levels in the young and middle aged and one could speculate that the phenomenon of lymphocyte subpopulation redistribution may be more complex and may involve other hormones such as TRH, TSH, GH, IGF1, monoamines such as melatonin, cytokines such as IL2 and chemokines (43-45). Aging of immune system function may be related to alteration of circadian rhythmicity, with loss of interaction among key lymphocyte subsets, immunomodulating hormones and cytokines/chemokines as well.

In conclusion, elderly people present anomalies of immune and endocrine parameters, represented by decrease of peripheral B-cell compartment, increase of activated T-cell compartment, decrease of TSH serum levels and alterations of circadian rhythmicity, expressed as loss, lower amplitude and phase delay or advance of a number of 24-hour rhythms, which may be responsible for altered interaction and function of the neuroendocrineimmune system.

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