

A pilot survey for circulatory diseases risk assessment in nuclear power plant workers

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ABSTRACT

Background: There are data suggesting that low dose radiation induced inflammatory reactions and changes of the immune system could be responsible for late circulatory and other chronic diseases. Chronic low dose radiation of occupationally exposed persons requires careful examination of their immune status. The purpose of this survey was to study some immunological parameters and serum proteins as suitable markers for screening cardiovascular diseases and chronic inflammatory state in NPP personnel. **Materials and Methods:** Lymphocyte populations were determined using four parameters by flow cytometer. Plasma levels of interleukin IL6, CRP and INF γ were determined by ELISA. **Results:** The main T lymphocyte populations did not show any differences to controls but there were trends of increasing activated CD3 HLA, CD4+25+ and CD8+38+ T lymphocytes and CRP and IL6 markers. Higher, but not significant averages were recorded for regulatory T lymphocytes probably due to their role in preventing of atherosclerosis. No dependence was established of the studied parameters to cardiovascular or other chronic diseases, a weak correlation was only recorded for IL6 with autoimmune ones ($p=0.042$). The results show that the age, obesity, and other lifestyle factors, particularly cigarette smoking could be considered as cofounder for circulatory diseases. **Conclusion:** It could be assume that radiation induced aging of T cells and activation of inflammatory response are partly involved in the development of inflammatory chronic diseases as the more pronounced deviations in the parameters are observed with increasing age and cumulative dose.

Keywords: lymphocyte populations, serum proteins, occupational radiation exposure, nuclear power plant workers.

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INTRODUCTION

Exposure of the heart to high doses of ionizing radiation has long been known to cause cardiac injury. Although some pathology can be observed early after irradiation, the heart is considered a late responding organ to radiation injury, a decade or more after exposure. Recently, clinical, epidemiological, and experimental studies have provided evidence that the cardiovascular system may also be injured at low doses of ionizing radiation. Some of the main cardiovascular effects are ischemic heart disease and stroke, potentially enhanced by an increased rate of hypertension⁽¹⁻⁴⁾. The

outcomes of ischemic heart disease and stroke in atomic bomb survivors and experimental studies suggest that vascular alterations may play a prominent role in the response to low-dose radiation⁽³⁻⁷⁾.

At the same time, an increasing number of epidemiological studies suggest that chronic low dose irradiation increases the risk of atherosclerosis⁽⁸⁻¹⁰⁾. Since the atherosclerosis is a chronic inflammatory disease which involves multiple types of immune cells, its pathogenesis has received considerable attention. The prevalent T population in atherosclerotic plaques are T helper (Th1) cells which include regulatory (CD4+CD25 + foxp3+)

T cells (Tregs) that maintain self-tolerance to autoantigens and suppress the activity of pro-atherogenic effector Th 17 cells ⁽¹¹⁾. Experimental studies and clinical trials have shown decline in Treg levels in atherosclerotic animals and in patients with coronary atherosclerosis and unstable angina, reveal that the low levels represent an independent risk factor for myocardial infarction and autoimmune diseases ^(12,13).

A few number of studies ^(2, 14) using cardiovascular biomarkers in asymptomatic patients indicate early signs of cardiovascular alterations even at a low dose. Health Protection Agency's AGIR in the United Kingdom 2010 ⁽¹⁵⁾, estimated a significantly elevated risk for ischemic heart disease and stroke for exposures above about 0.5 Gy (Gray). It was suggested that many inflammatory indices potentially relevant to circulatory disease could differentially be regulated below and above about 0.5 Gy. Well known is the role of cytokines in the inflammatory and immune responses. Since CRP (C reactive protein), interleukin IL6 and INF γ plasma levels are acute phase inflammatory mediators, they also could be used as informative markers of radiation-induced effects.

The purpose of this study was to investigate the risk of developing circulatory diseases in NPP personnel by some immunological parameters and serum proteins as markers for screening of cardiovascular diseases and chronic inflammatory state.

MATERIALS AND METHODS

The study was conducted under contracts between the National Centre of Radiobiology and Radiation Protection of the Ministry of Health of Bulgaria and the NPP Kozloduy, for health monitoring and study potential changes in the immunological status of NPP workers, exposed occupationally to low-dose radiation. Informed consent was obtained from all participants, according to the ethical standards of the responsible committee on human experimentation and with the Helsinki

Declaration of 1975, as revised in 2000. The radiation doses were determined by NPP individual exposure monitoring program.

Study subjects

The survey included two groups of men, one of 105 employees working in Nuclear Power Plant (NPP) Kozloduy Bulgaria, exposed to low and moderate doses of external γ ray irradiation and the other of 32 control persons at similar age and length of service without any work-related to ionizing radiation exposure.

For the purposes of our research the workers were divided in groups according to cumulative dose received as follows:

internal control; up to 25 mSv dose; up to 100 mSv dose; up to 200 mSv dose; and above 200 mSv dose (table 1).

Table 1. Personnel of NPP "Kozloduy" according to age, length of service and cumulative doses.

Personnel	Mean age (years)*	Length of service (years)*	Cumulative dose (mSv)*
Control – 32 persons	45.6 ± 8	19.5 ± 7	0
1 st group 27persons - 0.1–25 mSv	47.9 ± 6	21.5 ± 7	11.8 ± 9
2 nd group 31persons- 25.1–100 mSv	48.1 ± 8	22.6 ± 8	53.2 ± 21
3 rd group 19persons- 100.1–200 mSv	48.5 ± 3	25.2± 3	132.0 ± 30
4 th group 28persons - above 200 mSv	50.4 ± 6	27.7 ± 5	324.6 ± 89

* Standard deviation (±SD)

After filling a questionnaire, all participants have been subjected to medical examinations and underwent a basic hematological assay to evaluate their health status. Although no deviations in the basic laboratory tests and current infections were found in any of the respondents, some of them were diagnosed with cardiovascular diseases (hypertension and ischemic heart disease), obesity and other chronic diseases, whose distribution in the groups is illustrated in table 2.

The effect of smoking was evaluated by the immune parameters implemented in the study, as the investigation included 78 smokers and 54 nonsmokers.

Table 2. Health statuses of personnel of NPP "Kozloduy".

Diseases	Groups*				
	Control (%)	0-25 mSv (%)	25.1-100 mSv (%)	100.1-200 mSv (%)	>200 mSv (%)
Morbus Hyperthonicus	18.2	33.3	35.5	31.6	37.9
Cardiovascular diseases	21.2	33.3	35.5	36.8	39.9
Obesities	15.2	14.8	25.8	42.1	6.9
Autoimmune diseases	6.1	7.4	3.2	15.8	3.4
Hepatitis, not chronic, inactive	3.0	11.1	9.7	10.5	6.9
Viral diseases/ vaccination in the last month	15.2	14.8	6.5	10.5	10.3

* Data are presented as percentages from all individuals in each group

Experimental procedures

Four milliliters of blood was collected from each subject by venipuncture into Vacationer EDTA tubes (Greiner Bio-One GmbH, Kremsmunster, Austria). The blood cells were counted by automatic hematology analyzer ABX Pentra 60 C+ (HORIBA GROUP, France) operated in CBC + 5 DIFF (Cell Blood Count + 5 population differential count) modes.

Lymphocyte subpopulations were determined using a four-parameter flow cytometer in whole blood after lysis of erythrocytes by the method of Jackson ⁽¹⁶⁾. Staining of lymphocytes was carried out with a four-directly conjugated monoclonal antibodies, using the following dyes: fluorescein isothiocyanate (FITC), phycoerythrin (PE), pyridine chlorophyll protein (PerCP) and allophycocyanin (APC). Following tests were used: BD Multitest - CD3 / CD8 / CD45 / CD4 /, BD Multitest - CD8 / CD38 / CD3 / AntiMHLA-DR / BD Multitest - CD45RA / CD62L / CD3 / CD4 / (Becton Dickinson Biosciences, San Jose, California, USA). Human FoxP3 Buffer Set determines T regulatory CD4 + CD25 + FoxP3 lymphocytes, according to the attached protocol on Roncador G *et al.* ⁽¹⁷⁾. The stained samples analyzed by FACS Canto flow cytometer (Becton Dickinson Biosciences, San Jose, CA) in the application of Simulset and Cell Quest software evaluation of lymphocyte populations. Forward and right angle light scatter gated the lymphocyte fraction. Simulset software automatically collected a sufficient

number of events to obtain a minimum of 2000 lymphocytes within lymphocyte gate.

Plasma levels of interleukin IL6, CRP and INF γ were determined by ELISA method using kits of the GEN-PROBE Diaclone SAS, France.

Statistical analysis

SPSS v. 11.0.1 for Windows was used for data processing ⁽¹⁸⁾. Data were initially analyzed with Kolmogorov-Smirnov test to determine the degree of matching between two functions. It demonstrated the Gauss distribution of a group of variables.

Parametric methods were used for One-way analysis of variance (ANOVA). When the data in the different groups were not normally distributed, Mann-Whitney non-parametric method was applied to determine the credibility of the differences between two groups. The comparison of more than two groups was carried out by Kruskal-Wallis test. Nonparametric methods of Shapiro-Wilk, Mann-Witney and Kruskal-Wallis were applied. Variation analysis of quantitative variables was used- In all statistically reliable tests, differences with a probability of $p < 0.05$ were accepted. Correlation analysis - parametric (Pearson) and non-parametric (Spearman).

RESULTS

The relative and absolute values of cell parameters were analyzed. Data of the variation analysis of the studied parameters are presented in tables 3 to 5.

The parametric ANOVA test was applied to analyze CD3+ (percentage) and CD4+ (percentage) T lymphocytes, but for the other parameters, without normal distribution - Mann-Whitney и Kruskal-Wallis tests were used. No significant difference to the control and between groups was found, except for activated total CD 3 + HLADR + T lymphocytes, which showed a relatively higher average values in groups with cumulative doses above 100 mSv, significant in the third group (table 3). The correlation analysis revealed lack of any dependence with age, dose or length of service for total,

helper-inducer and suppressor-cytotoxic and activated CD8 + CD38 + T lymphocytes. Only activated total CD 3 + HLADR + T lymphocytes showed slightly positive correlation, significant with age (r=0.205 at p=0.026) and cumulative dose (r=0.187, at p = 0.037), while activated CD8 + CD38 + T lymphocytes revealed a weak negative dependence with age (r=-0.260 at p=0.004) and length of service (r=-0.229 at p=0.013).

The results of the variation analysis of CD4 + CD62L-, CD4 + CD62L+, activated CD4+25+ and regulatory CD4+25+FoxP3 T lymphocyte subpopulations are presented in table 4.

The coefficient of significance of examined immune parameters in all exposed groups revealed no statistical reliability as compared to controls (table 4). A tendency of reducing L-selectin-expressing subpopulation (CD4 + CD62L + T lymphocytes) was observed in groups with doses up to 200 mSv, whereas in the group with doses above 200 mSv average values were close to those of the control. The

increase of CD4 + CD25 + T lymphocytes, found in our previous study (16) was also confirmed in this investigation, although significant differences have been observed only between groups with lowest and highest cumulative doses (table 4). There was a slight positive but significant correlation of the percentages of these cells with age (r=0.249 at p=0.006), length of service (r=0.237 at p=0.011) and IL6 (r=0.233 with p=0.015 by Spearman). The results obtained for the regulatory T lymphocytes showed no statistically significant difference between exposed and control groups. There was a trend, not statistically significant, for increased mean with the cumulative dose, except for the third group. No dependence for this subpopulation was found with age, length of service and dose, except for a low positive correlation of the C reactive protein (r=0.211 with p=0.03 Pearson).

Results of CRP (C reactive protein), interleukin IL6 and INFγ plasma levels are presented in table 5.

Table 3. Variation analysis of CD3+, activated CD3+HLADR+, CD3+4+, CD3+8+ and activated CD8+38+relative to cumulative doses.

Parameters	Groups	X ± SD (%)	X ± SD (abs. values)
Total T lymphocytes (CD 3+)	Control	72.97 ± 6.7	1842.8 ± 594
	1 st 0.1 - 25 mSv	70.63 ± 7.3	1603.0 ± 494
	2 nd 25.1 - 100 mSv	74.87 ± 7.1	1806.8 ± 471
	3 rd 100.1 - 200 mSv	72.78 ± 8.0	1816.2 ± 503
	4 th above 200 mSv	72.79 ± 8.9	1917.68 ± 789
Activated total T lymphocytes (CD 3+HLADR+)	Control	6.37±3.9	150.47±80
	1 st 0.1 - 25 mSv	7.52±5.1	178.74±153
	2 nd 25.1 - 100 mSv	8.84±4.7	223.16±151
	3 rd 100.1 - 200 mSv	11.1±9.4 p = 0.05	278±237 p=0.041
	4 th above 200 mSv	9.62±7.6 p=0.093	257.34±214 p=0.055
Helper-inducer T lymphocytes (CD3+ CD4+)	Control	43.4±6.5	1094.28±353
	1 st 0.1 - 25 mSv	42.67±7.9	969.52±338
	2 nd 25.1 - 100 mSv	44.29±6.9	1074.9±299
	3 rd 100.1 - 200 mSv	43.0±10.4	1071.37±404
	4 th above 200 mSv	44.45±9.3	1168.48±395
Cytotoxic-suppressor T lymphocytes (CD3+ CD8+)	Control	28.47±7.1	720.94±288
	1 st 0.1 - 25 mSv	27.11±8.6	614.52±272
	2 nd 25.1 - 100 mSv	31.19±7.6	756.26±260
	3 rd 100.1 - 200 mSv	28.95±10.1	727.95±350
	4 th above 200 mSv	27.59±7.8	694.41±452
Activated Cytotoxic-suppressor T lymphocytes (CD8+CD38+)	Control	11.1 ± 5	279.3 ± 155
	1 st 0.1 - 25 mSv	11.7 ± 6	268.3 ± 154
	2 nd 25.1 - 100 mSv	11.8 ± 5	277.1 ± 107
	3 rd 100.1 - 200 mSv	10.7 ± 5	264.8 ± 131
	4 th above 200 mSv	12.8 ± 6	334.2 ± 202

Table 4. Variation analysis of CD4+CD62L-, CD4+ CD62L+, activated CD4+25+ and regulatory CD4+25+FoxP3 T lymphocytes relative to cumulative doses.

Parameters	Groups	X ± SD (%)	X ± SD (abs. values)
CD4+ CD62L- T lymphocytes	Control	11.63±4.1	290.88±160
	1 st 0.1 - 25 mSv	11.63±5.0	268.1±146
	2 nd 25.1 - 100 mSv	13.61±8.1	340.45±247
	3 rd 100.1 - 200 mSv	13.47±6.4	325.8±148
	4 th above 200 mSv	12.03±5.8	332.66±240
CD4+ CD62L+ T lymphocytes	Control	30.91±7.3	735.5±259
	1 st 0.1 - 25 mSv	29.67±7.8	669.0±259
	2 nd 25.1 - 100 mSv	29.48±6.4	742.3±312
	3 rd 100.1 - 200 mSv	26.26±9.7	658.9±308
	4 th above 200 mSv	30.83±8.9	771.7±239
Activated CD4 + CD25 + T lymphocytes	Control	13.09±6.4	242.35±132
	1 st 0.1 - 25 mSv	18.50±7.3 p = 0.004	341.04±233 p = 0.05
	2 nd 25.1 - 100 mSv	14.96±4.3	277.00±146
	3 rd 100.1 - 200 mSv	15.69±6.7	252.38±150
	4 th above 200 mSv	17.36±5.8 p = 0.012	313.72±181
Regulatory CD4+CD25+FoxP3 T lymphocytes	Control	6.00±3.2	61.32±28
	1 st 0.1 - 25 mSv	6.50±3.3	70.04±54
	2 nd 25.1 - 100 mSv	6.55±2.3	65.15±25
	3 rd 100.1 - 200 mSv	5.44±1.8	54.00±28
	4 th above 200 mSv	6.88±2.5	78.96±36

Table 5. Variation analysis on plasma levels of CRP, interleukin IL6 and INFγ to cumulative doses.

Groups	CRP (mg/l) X ± SD	IL6 (pg/ml) X ± SD	INFγ (pg/ml) X ± SD
Control	3.62 ± 4	1.97±2.0	15.3 ± 4.9
1 st 0.1 - 25 mSv	4.96 ± 4	1.98±1.9	14.1 ± 5.9
2 nd 25.1 - 100 mSv	4.56 ± 3.	2.39±1.4	13.4 ± 4.7
3 rd 100.1 - 200 mSv	5.65 ± 4	2.66±3.3	14.8 ± 7.2
4 th above 200 mSv	4.67 ± 4	2.27±1.4	14.1 ± 5.6

The protein's levels of IL 6, CRP and INFγ in control and exposed groups were analyzed and compared by Sigma Stat 3.5 program. The reference levels for the cytokines IL6 and INFγ was calculated on experimental data for the control group. After determining that the values of examined proteins had no Gauss distribution (Kolmogorov-Smirnov), the nonparametric methods of Mann-Whitney and Kruskal - Wallis were used.

As seen in Table 5, the results of the variation analysis of IL 6, CRP and INFγ displayed elevated averages compared to controls in all exposed groups, without statistical significance. The correlation analysis showed a low positive and significant correlations with dose (r=0.233

at p=0.010 Pearson), age (r=0.188 at p=0.044 Pearson) and length of service (r=0.232 at p=0.013 Pearson) for IL6, and absence of correlation of these variables for CRP and INFγ. For CRP was established weak positive but significant dependence of some T cells populations with CD3 + (r=0.270 at p=0.003 Pearson), CD3 + CD4 + (r=0.255 at p=0.006 Pearson) and CD4 + CD25 + Foxp3 T lymphocytes (r=0.211at p=0.03 Pearson). The same was found for IL6 with CD3 + percentage (r=0.204 at p=0.025 Spearman) and absolute values (r=0.234 at p=0.010 Spearman), CD4 + percentage values (r=0.195 at p=0.032 Spearman) and activated CD4 + CD25 + percentage values (r=0.233 at p=0.015 Spearman).

Regarding morbidity we have not found any relation of CRP with cardiovascular, autoimmune and virus diseases, whereas for IL6 was established relationship only with autoimmune diseases ($p=0.042$). Considering the impact on acute phase proteins and other factors of lifestyle, particularly cigarette smoking, a significant increase in CRP ($p=0.018$) and IL6 ($p<0.0001$) was determined in smokers.

There was no statistically significant difference for $\text{INF}\gamma$ related to cell-mediated immunity, despite slightly low average values for this cytokine in exposed groups. No correlation for $\text{INF}\gamma$ was found with age, dose, length of service, as well as with viral or chronic diseases.

DISCUSSION

The main T lymphocyte populations (total T lymphocytes, helper-inducer CD3+4+ suppressor-cytotoxic CD3+8+ T lymphocytes) in occupationally exposed NPP personnel did not show any differences to the control and dependence to cumulative doses, age and length of service. Other authors ^(19,20), as well as our previous studies ⁽²¹⁾ reported similar data for occupationally exposed persons. The variations found in activated total CD3 + HLADR + and in activated CD8 + 38 + T lymphocytes confirmed by the low correlations with dose and age, could be explained by the state of subclinical inflammation, which is more pronounced with increasing age and cumulative dose. Experimental data ⁽²²⁾ for increased IL2 (CD25) receptor expressing CD4+T lymphocyte population at low doses were in support to our previous results ⁽²³⁾. The studied regulatory CD4 + CD25 + Foxp3 + lymphocytes in the present survey demonstrated higher but not significant averages in most groups of exposed individuals. As CD4 +25+FoxP3 T regulatory lymphocytes are a subset of CD4+25+T activated lymphocytes, a question arises whether the increase in heterogeneous CD4 + CD25 + population was conditioned by the increase of regulatory T lymphocytes as part of this population. The results of both subpopulations,

suggest that the increase in the number of CD4 + CD25 +T lymphocytes reflect activation of immune response due to possible state of subclinical inflammation in part of the respondents.

In the present study, we also observed no significant, but higher plasma concentrations for C reactive protein and IL6, as well as a low correlation of interleukin IL6 with cumulative dose, age and length of service. These results are supported by data reports of Japanese authors ^(24, 25), who established increased plasma concentrations of inflammatory markers including C-reactive protein and pro-inflammatory cytokines IL6 and $\text{TNF } \alpha$, $\text{INF-}\gamma$ and IL-10 in the atomic bomb survivors.

Although the studied subpopulations of helper-inducer lymphocytes - CD4 + CD62L- and CD4 + CD62L + showed no significant differences to controls, a tendency was established in reducing L-selectin (CD62L) negative population and increasing L-selectin-positive at doses up to 200 mSv, while at higher doses reciprocal changes occurred. The observed relative decrease of L-selectin (CD62L) negative population could be explained by the greater radio sensitivity of CD62L negative populations ⁽²⁶⁾. It might be assumed that while the increase of L-selectin at doses up to 200 mSv reflects micro vascular activation of adhesion molecule expression ^(27,28), at higher doses a proteolytic release of L-selectin is going on. A dose-dependent increase of apoptosis in peripheral blood lymphocytes with a peak between 0.3 and 0.7 Gy was found experimentally, which is due to a time-dependent proteolysis ^(5, 6, 27, 29). This indicates that modulation of adhesion molecules may further contribute to the anti-inflammatory effect of low dose radiation ^(6, 29, 30).

The results of variation analysis of plasma proteins showed elevated averages for IL 6, CRP and slightly reduced $\text{INF}\gamma$ in all exposed groups. Shin *et al.* ⁽³¹⁾ found similar results for $\text{INF}\gamma$ after very low dose rates and doses of 0.2 Gy. The established in our study low positive correlations of CRP and IL6 with total, helper-inducer and activated T lymphocytes suggest a possible persistent antigenic challenge

in part of respondents. The possibility of ionizing radiation to induce a disorder in the regulation of pro-inflammatory cytokines increases the risk of many diseases such as cardiovascular, hypertension, autoimmune liver-related and thyroid diseases. The observed rising in regulatory T lymphocytes, although not significant, might be associated with a compensatory increase due to their role in preventing and delaying the progression of atherosclerosis, hypertension and circulatory disabilities as there was higher incidence of such disorders in the exposed individuals. Similar data for increase in regulatory T lymphocyte population in angiography medical staff is reported by Torkabadi *et al.* (19) and in persons survived the atomic bombing by Kusunoki *et al.* (25). In this respect it is important to emphasize the role of IL6 in maintaining the dynamic balance between regulatory CD4 + CD25 + Foxp3 + lymphocytes and TH17 lymphocyte population involved in pathogenesis of atherosclerosis. As IL6 directs differentiation of naïve helper-inducer lymphocytes to developing TH17 lymphocytes, its increased production could misbalance and increase the risk of autoimmune, chronic inflammatory and circulatory diseases (32). The low correlation between plasma levels of IL6 with activated CD4 + 25+, as well as the established dependence to the incidence of autoimmune diseases ($p=0.042$) could be in confirm to the above assumption.

The results of regulatory CD4 + 25+ FoxP3 T subpopulation revealed reduction in its average value only in the group with doses from 100 to 200 mSv, where the highest plasma concentrations of CRP and IL6 were detected. As shown in table 2, the largest proportion of individuals with obesity and higher incidence of hypertension, cardiovascular and autoimmune diseases was recorded in this group. Elevated CRP and IL6 levels have been consistently recorded in overweight and obese adults (33). The increase in adipose mass leads to adipocyte dysfunction and induction of pro-inflammatory mediators such as tumor necrosis factor- α , interleukin-6 (IL-6) and leptin (34). This might explain the decrease in regulatory T lymphocytes in the group with highest

frequency of obese persons, as their elevated levels of leptin could impair Treg proliferation (35).

An increase of IL6 and CRP in smokers, observed in the present study, suggests that smoking is likely to be an important co-founder of the association with circulatory disease.

In analyzing larger morbidity rate in groups with doses above 100 mSv, we should take into account that relatively high average age of this group also contribute to increased incidence of cardiovascular disease and hypertension, as these diseases are usually associated with aging processes. The obtained correlation with age of activated T lymphocyte populations, associated with chronic inflammation and acute phase proteins confirms such assumption.

The observed trends for increasing CRP and IL6 markers of inflammation and activated CD3 HLA, CD4+25+ and CD8+38+T lymphocytes, more pronounced with increasing age and cumulative dose, could be explained by the subclinical inflammation. The stated modulation of L selectin adhesion molecules and higher averages of regulatory T lymphocytes could be associated with activation of immune response and anti-inflammatory effects after low dose radiation. The obesity and other factors of lifestyle, particularly cigarette smoking could be also responsible for subclinical inflammation and considered as cofounder for circulatory diseases. Probably radiation induced aging of T cells accompanied by activation of inflammatory response are partly involved in the development of age-conditioned and inflammatory chronic diseases.

Conflicts of interest: Declared none.

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