Research Article

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Biomarkers in Medicine

Causes of variation in the neutrophil–lymphocyte and platelet–lymphocyte ratios: a twin-family study

Aim: Neutrophil–lymphocyte ratio (NLR) and platelet–lymphocyte ratio (PLR) are biomarkers for disease development, for whom little is known about causes of variation in the general population. **Materials & methods:** We estimated the heritability of PLR and NLR and examined their association with gender, demographic, lifestyle and environmental factors in a Dutch nonpatient twin family population (n = 8108). **Results:** Heritability was estimated at 64% for PLR and 36% for NLR. Men had on average higher NLR, but lower PLR levels than women. PLR and NLR increased significantly with age, decreased in colder months and showed small but significant sex- and age-specific associations with body composition and smoking. **Conclusion:** NLR and PLR levels are heritable and influenced by age, sex and environmental factors, such as seasonal conditions and lifestyle.

First draft submitted: 31 May 2016; Accepted for publication: 12 August 2016; Published online: 3 October 2016

Keywords: age • BMI • heritability • NLR • PLR • sex differences • smoking • weather conditions

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Hematological biomarkers in peripheral blood are indicators of physiological function and their levels may direct clinical decisions regarding disease status and treatment of patients. The two largest sets of immune cells, as reported in clinical hematological profiles, are neutrophils and lymphocytes. While both cell types play a key role in human inflammation and disease response, recent clinical studies suggest that their ratio may serve as a useful biomarker of disease. The neutrophil-to-lymphocyte ratio (NLR) has prognostic value for cancer progression [1,2], inflammatory disease [3,4] and cardiovascular disease [5]. A second hematological ratio of interest is the platelet-to-lymphocyte ratio (PLR), which has also been related to cancer progression [6], cardiovascular disease and inflammation [7].

To understand the role of NLR and PLR in disease processes, it is important to gain insight into the degree of variation in these ratios within nonpatient populations and the extent to which variation is due to genetic and nongenetic causes. Normal variation in immune function may be due to inherent factors such as age, sex and genetic constitution, environmental factors such as season, and lifestyle factors such as smoking and diet. To date, few studies examined the factors influencing the variation of NLR and PLR in nonpatient populations and most of those focused on NLR. Sex and age effects on NLR in the general population were examined in two studies [8,9], with similar results. No evidence was seen for sex differences in NLR but NLR did increase with increasing age. Li *et al.* [9] suggested that this age-related increase may reflect a higher prevalence of, often undetected, chronic infectious disease and cancer development in the older population. Genetic epidemiological studies of NLR and PLR are, to the best of our knowledge, lacking. However, genetic factors have been shown to contribute substantially to the phenotypic variation in neutrophil, lymphocyte and platelet counts, with heritability esti-

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mates of 67, 48–71% and 57–86%, respectively [10–13]. In addition to the contribution of inherent factors to variation among individuals, immune function may also be influenced by external factors. Seasonality is thought to be an important source of variation in the hematological profile [14]. Lymphocyte subset counts as well as platelet levels have been found to be lower in the summer season [15,16] and month-to-month changes in leukocyte and platelet levels were observed in a study of trained and untrained men [17]. Buckley *et al.* [16] estimated that seasonal factors accounted for 2% of the overall variation in platelet count, but not all studies show evidence for seasonal effects on platelet count [18]. Lifestyle may also contribute to variation in immunological function. Positive associations between BMI and NLR were observed in two nonpatient populations [8,9], but a third study did not find BMI to be related to NLR nor to PLR [19]. With respect to their subcomponents, larger waist circumference has been related to higher levels of lymphocytes, neutrophils and platelets [20], and these cell counts were also increased in obese women compared with nonobese women [21,22]. Smoking has also been related to increased NLR in two studies in the general population [8,23] and to increased neutrophil [23] and lymphocyte counts [24]. PLR, however, was not related to smoking [23] in the general population, and neither was platelet count in this study. Lack of evidence for an association between platelet count and smoking has been reported more often [25,26], though lower platelet levels in smokers have also been observed [27,28].

The present study analyzed data collected in over 8000 adult participants from The Netherlands Twin Register, including adult twins and their family members, who were very well characterized with respect to demographic and lifestyle traits and for whom information on date and time of blood sampling was available. Also, the study collected blood samples in women at a fixed moment of the menstrual cycle. We have two aims. Firstly, to estimate the contribution of the genome (heritability) and of nongenetic factors to variation in NLR and PLR and their subcomponents. Secondly, to further study nongenetic factors by examining the associations of the two ratios with age, sex, weather conditions at the day of sampling, CRP and IL6 levels as indicators of inflammation, and the influence of smoking behavior and BMI, although it should be recognized that some of these traits, like BMI or smoking, are themselves influenced by genes.

Materials & methods

Participants

Data for the present study came from participants in The Netherlands Twin Register Biobank projects, which took place between 2004 and 2008, and in 2011 [29–31]. After excluding outliers (i.e., absolute values exceeding mean ± 5 × standard deviation [SD]), NLR and PLR data were available for 9434 participants, clustered in 3411 families. In a next step, data were excluded in case of: illness in the week prior to blood sampling (n = 539); CRP ≥ 15 (n = 287); basophile count >0.02 × 10^9/L (n = 151); blood related disease or cancer (n = 83); and use of anti-inflammatory medication (n = 437); glucocorticoids (n = 143) or iron supplements (n = 28). This resulted in data for 8108 participants from 3411 families. The study protocol was approved by the Medical Ethics Committee of the VU University Medical Center Amsterdam (The Netherlands), and all participants provided informed consent.

General biobank procedure

Participants were visited at home, or in some cases at work, between 7 and 10:00 a.m. They were instructed to fast overnight and to refrain from smoking, heavy physical exertion and from medication use if possible in the morning prior to the visit. Fertile women without hormonal birth control were, if possible, seen on the 2nd to the 4th day of the menstrual cycle and women taking hormonal birth control were visited in their pill-free week. During the home visit, a brief interview was conducted concerning general health status, any chronic diseases, medication use and smoking history. Measures of height, weight, waist circumference and hip circumference were obtained. Peripheral venous blood samples were drawn by safety-lock butterfly needles in EDTA, lithium and sodium heparin, CTAD and PAX tubes. Immediately after blood collection, tubes were inverted several times to prevent clotting and subjected to initial processing in a mobile laboratory. Within 3–6 h after the blood draw all samples were transported to the laboratory facility in Leiden, The Netherlands (for details see [30,31]).

Blood parameters

Hematological profile

The 2 ml EDTA tubes were transported at room temperature to the laboratory, where the hematological profile was obtained using the Coulter system (Coulter Corporation, FL, USA). The profile consisted of total white blood cell count, percentages and numbers of neutrophils, lymphocytes, monocytes, eosinophils and basophils, red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width, platelet count and mean platelet volume.

NLR & PLR levels

NLR was calculated as absolute neutrophil count (10^9/L) divided by absolute lymphocyte count (10^9/L), and PLR was calculated as absolute platelet count (10^9/L) divided by absolute lymphocyte count (10^9/L).

CRP level

Plasma heparin was collected from a 9 ml heparin blood tube that was transported in melting ice to the laboratory. The plasma subsamples were snap-frozen and stored at -30°C. One heparin plasma subsample was used to determine CRP by the 1000 CRP assay (Diagnostic Product Corporation, CA, USA) [32].

IL6

EDTA plasma was obtained from the 9 ml EDTA tubes, which were stored in melting ice during transport. Upon arrival at the laboratory, the tubes were centrifuged for 20 min at 2000 × g at 4°C, and the plasma subsamples were snap-frozen and stored at -30°C. IL6 level was subsequently measured in one EDTA plasma subsample using the Quantikine Elisa Human IL-6 sR assay of R&D systems. Data were made missing if they exceeded mean ± 5 × SD (1.02% in total sample size) [33].

Health status, seasonal effects, BMI and smoking behavior

Health status

Participants were asked to report any chronic diseases and when they were last ill (i.e., less than 1 week ago, less than 1 month ago or more than 1 month ago). For any medication use, the dosage, brand and name were recorded.

BMI

BMI was calculated as weight (kg) divided by height squared (m^2).

Smoking behavior

Participants indicated whether they currently smoked or ever had smoked. If so, they were asked to provide information on the number of cigarettes smoked and how long they (had) smoked. Based on this information, participants were divided into five categories: nonsmoker, ex-smoker, light smoker (currently smoking less than ten cigarettes a day), average smoker (currently smoking 10–19 cigarettes a day) and heavy smoker (currently smoking 20 or more cigarettes a day).

Seasonal effects

The information on daily weather conditions was obtained from the website of the Royal Netherlands Meteorological Institute. We analyzed the daily data on temperature, wind speed, mean sea level, sunshine duration, global radiation and mean relative atmospheric humidity and potential evapotranspiration [34].

Analyses

For NLR and PLR, the contribution of genetic factors (heritability) was estimated based on the resemblance between relatives including mono- and dizygotic twins. First, we summarized familial resemblance with respect to NLR and PLR, corrected for age, sex and age × sex effects, by means of correlations. Next, genetic and nongenetic variance components were estimated by raw-data maximum likelihood in OpenMx [35]. The total variance in each phenotype was decomposed into four sources of variation: additive genetic (A), nonadditive genetic or dominance (D), common environmental (C) and unique environmental (E) variation. Common environmental variance was considered as the variance shared between siblings and twins (V_s) who grow up in the same family. The resemblance among family members was modeled as a function of A, D and C, making use of well-established genetic relatedness among family members. As monozygotic (MZ) twins derive from a single fertilized egg (zygote), they share approximately 100% of their genetic material, and consequently share all genetic (additive and dominance) variance. Dizygotic (DZ) twins, like full siblings, derive from two zygotes and share on average 50% of their segregating genes. Consequently, they share 50% of additive (V_A) and 25% of dominance genetic variance (V_D) [36]. Parents and offspring share exactly half of their genetic material, and share 50% of V_A, but no V_D. Our model allowed siblings and twins to share variance attributable to shared environment (V_S). Unshared influences (environmental, measurement error and personal mutations; V_E) contribute to total variance, but not to familial resemblance. We allowed for a correlation in phenotype between spouses (μ). Genetic analyses were done in twin families with at most one twin pair per family, and two brothers and two sisters, father and mother. The sample of 3251 families included 7481 participants (238 MZM, 99 DZM, 530 MZF, 215 DZF and 221 DOS complete twin pairs). Nested sub-models were compared with the full model by log-likelihood ratio test (-2LL), at a significance level of 0.05.

The association of NLR and PLR with IL6 and CRP was quantified by Pearson correlations in sex and age-corrected data. To test the effect of sex, we performed a *t*-test with sex as the independent factor on age-corrected NLR and PLR. The effects of age, temperature, smoking and BMI on NLR and PLR were tested by linear regression in STATA [37],

separately for men and women. All analyses were corrected for familial clustering using the option of robust cluster. All beta values presented below represent raw values and are evaluated at a significance level of 0.05.

Results

We carried out a series of analyses of the twin family data to gain insight into the heritability of PLR and NLR and their association with demographic factors, indicators of inflammation, seasonal conditions and lifestyle. Table 1 provides the descriptive statistics for NLR and PLR, their subcomponents neutrophil, lymphocyte and platelet count, and CRP and IL6 levels, separately for men and women. Table 2 contains the familial correlations for NLR and PLR. We found that the NLR and PLR familial correlations did not depend on sex (i.e., correlations in MZ males and MZ female twin pairs were equal, as were the correlations for male and female first-degree relatives; p = 0.23). For NLR, the MZ correlation was 0.36 (95% CI: 0.30–0.42) and the DZ correlation was 0.19 (0.16–0.22), which indicates an additive genetic model. For PLR, the MZ correlation was 0.64 (0.60–0.68), but the DZ correlation was at 0.24 (0.21-0.27) less than half the MZ correlation, suggesting the presence of nonadditive genetic effects. Spousal correlations were significant at 0.14 (0.07–0.21) for NLR and 0.17 (0.10–0.23) for PLR. The most parsimonious genetic models showed no evidence for common environmental influences on NLR (p = 0.47) and PLR (p = 0.99). The narrow sense heritability (proportion of total variance explained by additive genetic factors) of NLR was estimated at 35.8%, with no evidence for nonadditive effects. For PLR, the narrow sense heritability was 38.3%, with nonadditive effects accounting for an additional 25.9% of the total variance. The broad sense heritability for PLR was thus 64.2%. The remainder of the variance (64.2% in NLR and 35.8% in PLR) was explained by environmental factors. We also estimated the heritability for the three subcomponents of the ratios. The broad-sense heritability for neutrophil count was estimated at 41.1% (no nonadditive effects), for lymphocyte at 57.6% (22.4% due to nonadditive effects), and for platelet numbers at 70.5% (with 21.9% due to nonadditive effects). There was no evidence for common environmental effects for neutrophil count (p = 0.87), platelet count (p = 0.32) and lymphocyte count (p = 0.99).

For age- and sex-corrected values, the correlation between NLR and PLR was 0.49 (p < 0.001). We further determined the correlation of the two ratios with two established markers of inflammation, namely CRP and IL6. PLR correlated neither with CRP nor with IL6 (p > 0.05), but NLR correlated significantly with CRP (0.15; p < 0.001) and with IL6 (0.08; p < 0.001). NLR and PLR levels were affected by both sex and age. For age-corrected values, men had higher mean NLR levels than women (men, $Mean_{NLR}$ = 1.667; SE_{NLR} = 0.012; women, $Mean_{NLR}$ = 1.626; SE_{NLR} = 0.010; t[8106] = 2.2602; p = 0.009) and lower PLR levels than women (men, $Mean_{PLR}$ = 116.944; SE_{PLR} = 0.753; women, $Mean_{PLR}$ = 125.156; SE_{PLR} = 0.587; t[8106] = 20.073; p < 0.001). NLR increased with age in men but not in women (Table 3, 'Model 1'), while PLR increased with

Table 1. Mean (standard deviation) levels for the two ratios, their constituents, as well as the descriptive data for age, BMI, IL6, CRP and the percentage smokers in the twin family population, separately for men and women.

	Men	Women
n	3068	5040
Age (years)	44.13 (15.89)	43.07 (14.53)
NLR (%)	1.67 (0.66)	1.62 (0.70)
PLR (%)	117.11 (40.27)	125.05 (42.81)
Neutrophil (10^9/L)	3.41 (1.16)	3.45 (1.28)
Lymphocyte (10^9/L)	2.17 (0.63)	2.27 (0.71)
Platelet (10^9/L)	236.48 (53.36)	263.13 (6.84)
CRP† (mg/L)	2.01 (2.36)	2.66 (2.92)
IL6† (pg/mL)	1.69 (3.07)	1.64 (3.80)
BMI (kg/m^2)	25.47 (3.67)	24.84 (4.37)
Current smoker (% yes)	14.1	11.3

†The sample size for CRP: n = 3045 for men, n = 4980 for women; IL6: n = 2929 for men, n = 4867 for women.
NLR: Neutrophil–lymphocyte ratio; PLR: Platelet–lymphocyte ratio.

Table 2. Familial correlations and confidence intervals for neutrophil–lymphocyte ratio and platelet–lymphocyte ratio.

Pairs	NLR		PLR	
	R	95% CI	R	95% CI
MZ twins†	0.361	0.296–0.420	0.644	0.603–0.680
MZ male	0.396	0.277–0.496	0.607	0.518–0.675
MZ female	0.348	0.270–0.418	0.658	0.610–0.699
Male first-degree relatives†	0.186	0.111–0.258	0.223	0.142–0.299
DZ male	0.160	-0.078–0.392	0.295	0.085–0.461
Brother–male twin	0.331	0.199–0.439	0.342	0.028–0.557
Brother–brother	0.036	-0.225–0.309	0.308	0.122–0.450
Father–son	0.132	0.032–0.226	0.191	0.092–0.282
Female first-degree relatives†	0.172	0.127–0.216	0.240	0.199–0.279
DZ female	0.293	0.152–0.405	0.355	0.228–0.462
Sister–female twin	0.205	0.101–0.296	0.337	0.201–0.447
Sister–sister	0.179	0.083–0.266	0.241	0.150–0.327
Mother–daughter	0.141	0.079–0.198	0.221	0.165–0.275
Female–male first degree relatives†	0.205	0.165–0.244	0.240	0.197–0.282
DZ opposite sex	0.172	0.037–0.297	0.257	0.129–0.371
Brother–female twin	0.180	0.049–0.296	0.211	0.165–0.275
Sister–male twin	0.183	0.061–0.293	0.342	0.028–0.557
Sister–brother	0.127	0.006–0.240	0.217	0.102–0.322
Mother–son	0.237	0.149–0.317	0.261	0.173–0.340
Father–daughter	0.235	0.175–0.296	0.233	0.172–0.291
Parents (father–mother)†	0.137	0.066–0.207	0.166	0.101–0.230
Heritability†	0.358	0.304–0.421	0.642	0.598–0.683

†Correlations were obtained from a sub-models, in which the correlations for the subgroups of family relations, which were tested, were set to be equal.
DZ: Dizygotic; MZ: Monozygotic; NLR: Neutrophil–lymphocyte ratio; PLR: Platelet–lymphocyte ratio.

age in both men and women (see Table 3, 'Model 1').

Next, we explored the influence of seasonal conditions on variation in the ratios. Figure 1 illustrates the association between daily temperature and age-corrected NLR and PLR for men and women. To avoid outliers due to periods with very few observations, we restricted the entries in the graph to the months with more than 75 data points between August 2004 and December 2007. We note a similar pattern for NLR and PLR from year to year: Overall, NLR and PLR ratios increase with decreasing temperature. This pattern seems more evident in the female group than in the male group. To formally test for the effect of temperature, we included this variable in a regression analysis conducted separately by sex and taking age into account. The results, shown in Table 3 (Model 2), demonstrate that both NLR and PLR are negatively significantly associated with daily temperature in women, but not in men. There was no evidence for significant age x temperature interactions for NLR and PLR.

We also explored the associations of NLR and PLR levels with the other weather-related information available. Although sunshine duration, global radiation, atmospheric humidity and evapotranspiration were related to NLR and PLR, these associations were rendered insignificant by the addition of temperature. One exception was the effect of global radiation on NLR: as the daily global radiation level increased, NLR levels decreased ($\beta = 2.01E\text{-}5$; $p < 0.001$).

Table 4 includes the average NLR and PLR values as a function of smoking, BMI and sex, while Table 3 includes the results of the linear regression modeling (see 'Model 3' in Table 3). Smoking was not significantly associated with NLR in either men or women. BMI was not associated with NLR in men, but it was related to NLR in women. In women, NLR increased with increasing BMI and there was a significant age × BMI interaction, due to an alleviation of the BMI

Table 3. Results of the linear regression modeling for neutrophil–lymphocyte ratio and platelet–lymphocyte ratio, separate for men and women.

Variables	Men						Women					
	NLR			PLR			NLR			PLR		
	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3
Age (years)	0.0105***	0.0092***	0.009	0.2824***	0.2723*	1.3813***	-0.0000	-0.0008	0.0083	0.2455***	0.1062	1.4980***
Temperature (0.1°C)		-0.0000	-0.0002		-0.0481	-0.0352		-0.0123*	-0.0014*		-0.1083***	-0.0991**
Age × temperature		0.0000	0.0000		0.0000	0.000		0.000	0.0000		0.0012	0.0000
BMI			-0.0023			0.496			0.0362***			2.1353***
Age × BMI			0.0000			-0.0350**			-0.0004*			-0.0524***
Smoking			0.0501			-3.7760*			-0.0091			-5.5355***
Age × smoking			0.0000			-0.0640			0.0000			-0.0471
N	3068	3068	3040	3068	3068	3040	5040	5040	4980	5040	5040	4980
R^2	0.0580	0.0582	0.0608	0.0124	0.0168	0.0611	0.0001	0.0048	0.0162	0.0069	0.0137	0.0571

*p < 0.05.
**p < 0.01.
***p < 0.001.
NLR: Neutrophil–lymphocyte ratio; PLR: Platelet–lymphocyte ratio.

association with increased age. PLR was more strongly affected by smoking and BMI. In women, there was a significant BMI main effect as well as an age × BMI interaction: the positive association was reduced at older age. Though we had limited numbers of participants at older ages, an exploration of the data seems to suggest the direction of event may be even reversed at old age. A similar pattern, though less strong, was seen for the men. Unexpectedly, smoking was associated with a decrease in PLR in both men and women, while age × smoking interaction effects were not present. To explore the mechanisms underlying the association with smoking, we also examined the relation between the subcomponents and smoking. Smoking was related to an increase in neutrophils ($\beta = 0.305$; $p < 0.001$) and lymphocytes ($\beta = 0.260$; $p < 0.001$), but had no significant effect on platelets ($\beta = 0.017$; $p = 0.133$). There was no evidence for smoking × age interactions for the subcomponents.

The full model (Table 3, 'Model 3') including age, temperature, BMI, smoking and their interactions with age, explained about 6% of the variance in PLR in both men and women. In men, this model also explained around 6% of the variance for NLR, but in women only 1.6% of the variance in NLR was explained by the factors included in the model.

Discussion

The current study examined causes of variation in NLR and PLR to provide an insight into individual differences in these biomarkers in the nonpatient population. We examined the effects of genetics, demographics, seasonal conditions and lifestyle and described for the first time the importance of genetic factors for variation in PLR and NLR. Especially PLR is influenced to a large extent (64%) by additive and nonadditive genetic influences. This high heritability is in accordance with the heritability estimates reported for the individual platelets and lymphocytes components, which ranged from 48 to 86% in previous studies [10–13] and which we here estimated to be 71% for platelets and 58% for lymphocytes. Genetic factors also explain the variation in NLR with heritability estimated at 36%. A lower heritability was also observed for neutrophil count (38%). The genetic architecture underlying NLR and PLR was similar in men and women. Also, there were no differences between the generations in the genetic architecture of NLR and PLR as indicated by similar correlations for parents and offspring as for siblings. Our data showed significant spousal associations, which is in line with previous reports of assortative mating for immune parameters [38] but may also reflect a shared spousal environment leading to a similar immune response.

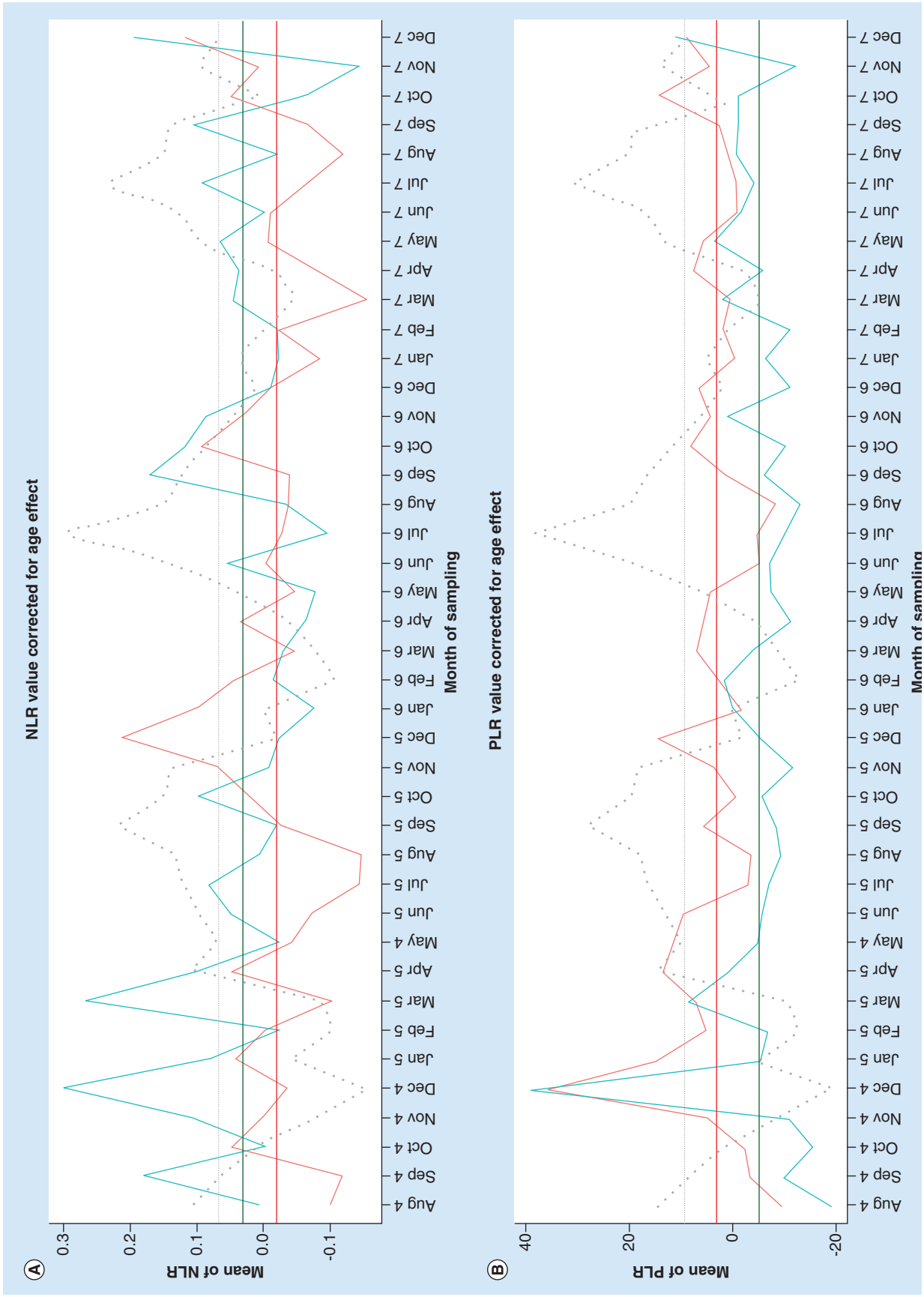

Figure 1. The relationship between monthly temperature and the average neutrophil–lymphocyte ratio and platelet–lymphocyte ratio, for men and women separately.
The relationship between monthly temperature (gray dotted line) and the average NLR and PLR for men (blue line) and women (red line).
NLR: Neutrophil–lymphocyte ratio; PLR: Platelet–lymphocyte ratio.

Table 4. Age-corrected mean (standard error) neutrophil–lymphocyte ratio and platelet–lymphocyte ratio as a function of BMI and smoking category, for men and women separately.

	Men			Women		
	N	NLR	PLR	N	NLR	PLR
Underweight	63	1.764 (0.082)	126.569 (5.097)	188	1.555 (0.051)	125.943 (4.120)
Normal	1404	1.660 (0.017)	119.264 (1.089)	2791	1.569 (0.013)	125.418 (0.817)
Overweight	1372	1.676 (0.018)	115.797 (1.100)	1594	1.689 (0.018)	124.749 (1.085)
Obesity	216	1.667 (0.044)	108.952 (2.735)	436	1.763 (0.034)	123.169 (2.058)
Never-smoker	1268	1.655 (0.019)	121.610 (1.147)	2669	1.630 (0.014)	129.469 (0.813)
Ex-smoker	1021	1.626 (0.021)	12.297 (1.310)	1377	1.576 (0.019)	127.758 (1.143)
Light smoker	330	1.737 (0.035)	114.615 (2.160)	392	1.656 (0.035)	118.092 (2.111)
Average smoker	239	1.743 (0.041)	97.758 (2.543)	378	1.668 (0.036)	104.433 (2.149)
Heavy smoker	193	1.785 (0.046)	97.890 (2.825)	193	1.724 (0.051)	98.335 (3.007)

For the purpose of the overview, BMI was classified in four categories: underweight (BMI <18.5), normal (18.5–24.9), overweight (25.0–29.9) and obese (BMI ≥30).
NLR: Neutrophil–lymphocyte ratio; PLR: Platelet–lymphocyte ratio.

In addition to being significantly influenced by our genome, normal variation in NLR and PLR levels is also explained by differences in gender, age, and environmental and lifestyle traits. There were sex differences in mean levels, with higher NLR and lower PLR in men compared with women, and an older age was related to an increase in PLR and, to a lesser extent, to an increase in NLR. As suggested by Li *et al.* [9], the age effect could reflect underlying diseases in the older population, even though we selected relatively healthy individuals as determined by immunological data, medication use and disease reports. It is possible that some diseases were present at sub-threshold level and the higher prevalence of autoimmune disease in women, especially after age 50, is well established [39].

Nongenetic causes of variance in NLR and PLR included the effect of weather conditions. For both ratios, average levels were higher in colder months, indicating seasonal influences on immune parameters. This is consistent with previous work, which found higher levels of inflammation during the winter in European countries [40] and in line with previous studies on seasonal effects on cell counts in humans [14–17]. One likely explanation for the higher levels during the winter is that there is a higher prevalence of viral infections during the cold season [18], though many other factors are likely involved in the seasonal effects on immune response and further study is needed [40]. Women may be more sensitive to the seasonal changes, as an effect of temperature was mainly visible in women.

Lifestyle factors were related to individual differences in the two ratios. In women, a higher BMI was related to higher NLR and PLR levels and there was also evidence for significant age × BMI interactions for both ratios. In men, NLR was not influenced by BMI but there was a significant age × BMI interaction for PLR. The interactions with age were due to the fact that the influence of BMI became less strong at an older age. Our data suggested there may even be a reduction in the ratios at old age, and studies including more participants in the old-age range are needed to confirm this. A positive association between BMI and NLR has been found before [8,9] and obesity is often considered to be associated with a chronic state of inflammation [41]. Dietary habits may also influence both platelet and leukocyte counts [42]. The greater influence of BMI at younger ages points to the importance of weight control in early life.

Smoking increased both neutrophil and lymphocyte count, but whereas we observed decreased PLR levels in men and women who smoked, there was no effect of smoking on NLR. Tulgar *et al.* [23] found no effect of smoking on PLR or its subcomponents, but this may be due to a small sample size, as the descriptive data do suggest a lower average PLR in smokers compared with nonsmokers. This study also reported NLR to be increased in smokers, as did a larger study [8]. The mechanisms underlying the association of NLR and PLR with disease are not fully understood. Increases in NLR and PLR may be indicative of a decreased ability to detect and destroy infected cells, and of increased tumor-promoting activities. A higher NLR indicates a shift in the balance between neutrophil and lymphocytes, which in our sample was due to both a decrease in lymphocyte count and an increase in neutrophil count. Lower lymphocyte counts are associated with poorer survival in different types of cancer [43,44], while high lymphocyte counts are related to better responses to cytotoxic treatment and to better prognosis in cancer patients [45]. Neutrophils have also been reported

to secrete tumor growth promoting factors, including vascular endothelial growth factor, hepatocyte growth factor, multiple interleukins and matrix metalloproteinases, and may thus contribute to a tumor stimulating microenvironment [46,47]. A high BMI seems to be related to an increased imbalance between lymphocytes and neutrophils, resulting in an increased NLR, especially in women. With respect to PLR, overall, higher PLR in our study was related to lower lymphocyte and higher platelet numbers. Platelets play an important role in angiogenesis, thrombosis and hemostasis and increased platelet numbers have been implicated in the development of cardiovascular disease [48] and cancer progression [49]. Further study of the relationship of the two ratios with smoking and BMI in a longitudinal sample, with attention to sex differences and interactions, may provide important information about the way lifestyle influences our health.

Several studies have suggested that NLR and PLR may also be used as indicators of inflammation and provide a cheap and easily-obtainable alternative to the currently used CRP and cytokines, such as IL6 [50]. However, the low correlations we observed between the ratios and these two inflammatory markers argue against this. Correlations may have been low because of exclusion criteria in our study, which included high CRP levels. Upon exploring the correlations in the total sample, the correlations for NLR with CRP and IL6 were not much higher (0.214 and 0.121, respectively) while PLR remained unrelated to CRP and IL6. Our results agree with those of Oh *et al.* [51], in that NLR and PLR are no replacements for CRP and IL6 but should be used in addition to each other.

The correlation between NLR and PLR in our healthy population was moderate (r = 0.49). The presented differences in heritability, in the effects of lifestyle and in the association with IL6 and CRP, indicate that the mechanisms underlying individual differences in the two ratios are not the same for NLR and PLR. This is in line with studies showing that the two ratios do not predict disease progress to the same extent [52] and may act as independent disease predictors [53].

The combination of demographic and seasonal factors, smoking and BMI explained around 6% of the variation in NLR and PLR. This is substantially smaller than the part of the variance explained by genetic factors; 36% in NLR and 64% in PLR. Thus, it is of importance to realize that variation in NLR and PLR to a large extent can represent genetic variation, and that high levels in these ratios also may occur independent of disease status. While a further search for additional environmental factors influencing variation in these immune parameters is warranted, more insight into the genes and genetic mechanisms underlying the high heritability is needed and gene finding studies form an important next step in characterizing the DNA polymorphisms causing variation in NLR and PLR.

In conclusion, variation in basal NLR and PLR in a general population sample is influenced by the genome, by age and sex, by lifestyle factors and by environmental factors, such as seasonal weather conditions.

Conclusion

This first study on the heritability of NLR and PLR showed that genetic factors influence variation in NLR, and to an even larger extent, in PLR. To provide more insight into the genetic variation in NLR and PLR, gene finding studies are needed. Nongenetic factors are more relevant to NLR than to PLR and while sex, age, seasonal conditions and lifestyle play a role, these factors explain only a small part of the variation. For NLR in particular, studies are warranted to identify additional environmental influences.

Acknowledgements

The authors thank all the twin families who participated in The Netherlands Twin Register Biobank project.

Financial & competing interests disclosure

This work was supported by: Genotype/phenotype database for genetic studies (ZonMW Middelgroot [911-09-032]); Database Twin register (NWO 575-25-006); Twin family database for behavior genetics and genomics studies (NWO 480-04-004); Genome-wide analyses of European twin and population cohorts (EU/QLRT-2001-01254); a collaborative study of the genetics of DZ twinning (NIH R01 HD042157-01A1); EMGO+ Institute for Health and Care Research, Neuroscience Campus Amsterdam, Center for Medical Systems Biology (CMSB), Biobanking and Biomolecular Resources Research Infrastructure (BBMRI-NL) 184.021.007; GENOMEUTWIN/EU (QLG2-CT-2002-01254); NIH (NIHHEALTHF4-2007-201413); European Research Council (230374-GMI). B Lin received a PhD grant (201206180099) from the China Scholarship Council. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Background

- Neutrophil–lymphocyte ratio (NLR) and platelet–lymphocyte ratio (PLR) are biomarkers for disease development, but there is variation among individuals that is not attributable to disease status, and which may reflect genetic variation in NLR and PLR.
- We examined to what degree variation in NLR and PLR can be explained by genetic (heritability) and nongenetic factors.
- We also characterized some of the nongenetic factors by examining the association of NLR and PLR with gender, demographic, lifestyle and environmental factors.

Methods

- After applying outlier and disease-related exclusion criteria, data on NLR and PLR were available for 8108 twins and their family members.
- Twin-family data were analyzed by genetic structural equation modeling to estimate the heritability of PLR and NLR.
- Associations of NLR and PLR with age, smoking behavior, BMI and seasonal conditions were tested using linear regression.

Results

- Broad-sense heritability was estimated at 36% for NLR and 64% for PLR, with nonadditive genetic effects evident for PLR.
- The correlation between NLR and PLR was 0.49; $p < 0.001$. NLR, but not PLR, correlated significantly with CRP (0.15; $p < 0.001$) and with IL6 (0.08; $p < 0.001$).
- Men had higher mean NLR levels than women and lower PLR levels than women.
- PLR, and to a lesser extent NLR, increased significantly with age.
- Seasonal differences were sex-specific: in colder months average NLR and PLR were higher in women, but not in men.
- Smoking was not significantly associated with NLR, but was associated with a decrease in PLR in both men and women.
- A high BMI was related to higher levels of NLR and PLR in women, though the association was less strong at higher ages. In men the BMI effect was only evident for PLR and age-related.

Discussion

- PLR, and to a lesser extent, NLR are heritable traits.
- NLR and PLR are influenced by age, sex, seasonal conditions and lifestyle factors.
- The effects of seasonal factors and lifestyle factors may be age- and sex-dependent.
- The mechanisms underlying individual differences in NLR and PLR are not the same.
- More studies are needed to increase our knowledge about environmental influences on NLR and PLR.
- This work leads to the next steps of finding the genes involved in variance in NLR and PLR.

References

Papers of special note have been highlighted as:
• of interest; •• of considerable interest

1 Hu K, Lou L, Ye J, Zhang S. Prognostic role of the neutrophil-lymphocyte ratio in renal cell carcinoma: a meta-analysis. *BMJ Open* 5(4), e006404 (2015).

2 Hu Zd, Huang Yl, Qin BD *et al.* Prognostic value of neutrophil to lymphocyte ratio for gastric cancer. *Ann. Transl. Med.* 3(4), 50–58 (2015).

3 Sarikaya M, Dogan Z, Ergul B, Filik L. Neutrophil-to-lymphocyte ratio as a sensitive marker in diagnosis of celiac disease. *Ann. Gastroenterol.* 27(4), 431–432 (2014).

4 Acarturk G, Acay A, Demir K, Ulu MS, Ahsen A, Yuksel S. Neutrophil-to-lymphocyte ratio in inflammatory bowel disease – as a new predictor of disease severity. *Bratisl. Lek. Listy* 116(4), 213–217 (2015).

5 Shah N, Parikh V, Patel N *et al.* Neutrophil lymphocyte ratio significantly improves the Framingham risk score in prediction of coronary heart disease mortality: insights from the National Health and Nutrition Examination Survey-III. *Int. J. Cardiol.* 171(3), 390–397 (2014).

6 Zhou X, Du YP, Huang ZB *et al.* Prognostic value of PLR in various cancers: a meta-analysis. *PLoS ONE* 9(6), e101119 (2014).

7 Ozturk C, Balta S, Balta I *et al.* Neutrophil–lymphocyte ratio and carotid-intima media thickness in patients with Behcet disease without cardiovascular involvement. *Angiology* 66(3), 291–296 (2015).

8 Azab B, Camacho-Rivera M, Taioli E. Average values and racial differences of neutrophil lymphocyte ratio among a nationally representative sample of United States subjects. *PLoS ONE* 9, e112361 (2014).

• **The first paper to study variance in neutrophil–lymphocyte ratio (NLR) in a large nonpatient population.**

9 Li J, Chen QY, Luo XH *et al.* Neutrophil-to-lymphocyte ratio positively correlates to age in healthy population. *J. Clin. Lab. Anal.* 29(6), 437–443 (2015).

• **A detailed examination of NLR in the general population.**

10 Evans DM, Frazer LH, Martin NG. Genetic and environmental causes of variation in basal levels of blood cells. *Twin Res.* 2(4), 250–257 (1999).

• **A good start for those interested in genetic influences on cell counts.**

11 Garner C, Tatu T, Reittie JE *et al.* Genetic influences on F cells and other hematologic variables: a twin heritability study. *Blood* 95(1), 342–346 (2000).

12 Hall MA, Ahmadi KR, Norman P *et al.* Genetic influence on peripheral blood T lymphocyte levels. *Genes Immun.* 1(7), 423–427 (2000).

13 Whitfield JB, Martin NG. Genetic and environmental influences on the size and number of cells in the blood. *Genet. Epidemiol.* 2(2), 133–144 (1985).

14 Maes M, Stevens W, Scharpe S *et al.* Seasonal variation in peripheral blood leukocyte subsets and in serum interleukin-6, and soluble interleukin-2 and -6 receptor concentrations in normal volunteers. *Experientia* 50(9), 821–829 (1994).

15 Paglieroni TG, Holland PV. Circannual variation in lymphocyte subsets, revisited. *Transfusion* 34(6), 512–516 (1994).

16 Buckley MF, James JW, Brown DE *et al.* A novel approach to the assessment of variations in the human platelet count. *Thromb. Haemost.* 83(3), 480–484 (2000).

17 Broadbent S. Seasonal changes in haematology, lymphocyte transferrin receptors and intracellular iron in Ironman triathletes and untrained men. *Eur. J. Appl. Physiol.* 111(1), 93–100 (2011).

18 Crawford VL, McNerlan SE, Stout RW. Seasonal changes in platelets, fibrinogen and factor VII in elderly people. *Age Ageing* 32(6), 661–665 (2003).

• **An introduction regarding the seasonal influences on immune function.**

19 Furuncuoğlu Y, Tulgar S, Dogan AN, Cakar S, Tulgar YK, Cakiroglu B. How obesity affects the neutrophil/lymphocyte and platelet/lymphocyte ratio, systemic immune-inflammatory index and platelet indices: a retrospective study. *Eur. Rev. Med. Pharmacol. Sci.* 20(7), 1300–1306 (2016).

• **A detailed investigation of the influence of obesity on NLR as well as platelet–lymphocyte ratio (PLR) in nonpatients.**

20 Vuong J, Qiu Y, La M, Clarke G, Swinkels DW, Cembrowski G. Reference intervals of complete blood count constituents are highly correlated to waist circumference: should obese patients have their own "normal values?" *Am. J. Hematol.* 89(7), 671–677 (2014).

21 Farhangi MA, Keshavarz SA, Eshraghian M, Ostadrahimi A, Saboor-Yaraghi AA. White blood cell count in women: relation to inflammatory biomarkers, haematological profiles, visceral adiposity, and other cardiovascular risk factors. *J. Health Popul. Nutr.* 31(1), 58–64 (2013).

22 Samocha-Bonet D, Justo D, Rogowski O *et al.* Platelet counts and platelet activation markers in obese subjects. *Mediators Inflamm.* 2008, 834153 (2008).

23 Tulgar YK, Cakar S, Tulgar S, Dalkilic O, Cakiroglu B, Uyanik BS. The effect of smoking on neutrophil/lymphocyte and platelet/lymphocyte ratio and platelet indices: a retrospective study. *Eur. Rev. Med. Pharmacol. Sci.* 20(14), 3112–3118 (2016).

24 Andreoli C, Bassi A, Gregg EO, Nunziata A, Puntoni R, Corsini E. Effects of cigarette smoking on circulating leukocytes and plasma cytokines in monozygotic twins. *Clin. Chem. Lab. Med.* 53(1), 57–64 (2015).

25 Suwansaksri J, Wiwanitkit V, Soogarun S. Effect of smoking on platelet count and platelet parameters: an observation. *Clin. Appl. Thromb. Hemost.* 10(3), 287–288 (2014).

26 Mercelina-Roumans PE, Ubachs JM, van Wersch JW. Platelet count and platelet indices at various stages of normal pregnancy in smoking and non-smoking women. *Eur. J. Clin. Chem. Clin. Biochem.* 33(5), 267–269 (1995).

27 Varol E, Icli A, Kocyigit S, Erdogan D, Ozaydin M, Dogan A. Effect of smoking cessation on mean platelet volume. *Clin. Appl. Thromb. Hemost.* 19(3), 315–319 (2013).

28 Green MS, Peled I, Najenson T. Gender differences in platelet count and its association with cigarette smoking in a large cohort in Israel. *J. Clin. Epidemiol.* 45(1), 77–84 (1992).

29 Willemsen G, Vink JM, Abdellaoui A *et al.* The Adult Netherlands Twin Register: twenty-five years of survey and biological data collection. *Twin. Res. Hum. Genet.* 16(1), 271–281 (2013).

30 Willemsen G, De Geus EJ, Bartels M *et al.* The Netherlands Twin Register biobank: a resource for genetic epidemiological studies. *Twin. Res. Hum. Genet.* 13(3), 231–245 (2010).

31 Sirota M, Willemsen G, Sundar P *et al.* Effect of genome and environment on metabolic and inflammatory profiles. *PLoS ONE* 10(4), e0120898 (2015).

32 Neijts M, Van Dongen J, Kluft C, Boomsma DI, Willemsen G, De Geus EJ. Genetic architecture of the pro-inflammatory state in an extended twin-family design. *Twin. Res. Hum. Genet.* 16(5), 931–940 (2013).

• **Heritability estimates for TNF-α, IL6, C-reactive protein and fibrinogen levels based on a large study of twins and their family members.**

33 Van Dongen J, Jansen R, Smit D *et al.* The contribution of the functional IL6R polymorphism rs2228145, eQTLs and other genome-wide SNPs to the heritability of plasma sIL-6R levels. *Behav. Genet.* 44(4), 368–382 (2014).

•• **An excellent tutorial on methods in genetic epidemiology and genetic linkage and association studies applied to the analysis of variation in IL6 receptor levels.**

34 KNMI, Daily data on the weather in the Netherlands. 2004–2008 www.knmi.nl/nederland-nu/klimatologie/daggegevens

35 Boker S, Neale M, Maes H *et al.* OpenMx: an open source extended structural equation modeling framework. *Psychometrika* 76(2), 306–317 (2011).

36 Falconer DS, Mackay TFC. *Introduction to Quantitative Genetics.* Longman, Essex, UK, 174–176 (1996).

37 Statacorp. Stata Statistical Software: Release College Station, TX: StataCorp LP (2013). www.stata.com/

38 Havlicek J, Roberts SC. MHC-correlated mate choice in humans: a review. *Psychoneuroendocrinology* 34(4), 497–512 (2009).

39 Fairweather D, Frisancho-Kiss S, Rose NR. Sex differences in autoimmune disease from a pathological perspective. *Am. J. Pathol.* 173(3), 600–609 (2008).

40 Dopico XC, Evangelou M, Ferreira RC *et al.* Widespread seasonal gene expression reveals annual differences in human immunity and physiology. *Nat. Commun.* 6, 7000 (2015).

•• **For those who want to understand more about the complex mechanisms linking genetic and seasonal influences.**

41 Yudkin JS, Kumari M, Humphries SE, Mohamed-Ali V. Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? *Atherosclerosis* 148(2), 209–214 (2000).

42 Bonaccio M, Di Castelnuovo A, De Curtis A *et al.* Adherence to the Mediterranean diet is associated with lower platelet and leukocyte counts: results from the Moli-sani study. *Blood* 123(19), 3037–3044 (2014).

43 Fogar P, Sperti C, Basso D *et al.* Decreased total lymphocyte counts in pancreatic cancer: an index of adverse outcome. *Pancreas* 32(1), 22–28 (2006).

44 Liang L, Zhu J, Jia H *et al.* Predictive value of pretreatment lymphocyte count in stage II colorectal cancer and in high-risk patients treated with adjuvant chemotherapy. *Oncotarget* 7(1), 1014–1028 (2016).

45 Di Caro G, Bergomas F, Grizzi F *et al.* Occurrence of tertiary lymphoid tissue is associated with T-cell infiltration and predicts better prognosis in early-stage colorectal cancers. *Clin. Cancer Res.* 20(8), 2147–2158 (2014).

46 Mccourt M, Wang JH, Sookhai S, Redmond HP. Proinflammatory mediators stimulate neutrophil-directed angiogenesis. *Arch. Surg.* 134(12), 1325–1331, discussion 1331–1322 (1999).

47 Di Carlo E, Forni G, Musiani P. Neutrophils in the antitumoral immune response. *Chem. Immunol. Allergy* 83, 182–203 (2003).

48 Thaulow E, Erikssen J, Sandvik L, Stormorken H, Cohn PF. Blood platelet count and function are related to total and cardiovascular death in apparently healthy men. *Circulation* 84(2), 613–617 (1991).

49 Franco AT, Corken A, Ware J. Platelets at the interface of thrombosis, inflammation, and cancer. *Blood* 126(5), 582–588 (2015).

50 Imtiaz F, Shafique K, Mirza SS, Ayoob Z, Vart P, Rao S. Neutrophil lymphocyte ratio as a measure of systemic inflammation in prevalent chronic diseases in Asian population. *Int. Arch. Med.* 5(1), 2 (2012).

51 Oh BS, Jang JW, Kwon JH *et al.* Prognostic value of C-reactive protein and neutrophil-to-lymphocyte ratio in patients with hepatocellular carcinoma. *BMC Cancer* 13, 78 (2013).

52 Xia W-K, Liu Z-L, Shen D, Lin Q-F, Su J, Mao W-D. Prognostic performance of pre-treatment NLR and PLR in patients suffering from Osteosarcoma. *World J. Surg. Oncol.* 14, 127 (2016).

53 Koh C-H, Bhoo-Pathy N, Ng K-L *et al.* Utility of pre-treatment neutrophil–lymphocyte ratio and platelet–lymphocyte ratio as prognostic factors in breast cancer. *Br. J. Cancer* 113, 150–158 (2013).