CHAPTER 9

Active smoking and macrocytosis in the general population: Two populationbased cohort studies

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To the Editor:

Macrocytosis, an elevated mean corpuscular volume (MCV) of erythrocytes, is a highly prevalent phenomenon in adult individuals (1). MCV is the measurement of the average volume of red blood cells, and macrocytosis is defined as a MCV exceeding 100 fL. Currently, in textbooks and guidelines a myriad causes are being mentioned for macrocytosis, with vitamin B12 and folate deficiency, alcohol use, myeloid dysplastic syndromes, and liver disease as the most prominent ones (2). In the 70s, a number of papers have reported a positive association between smoking and MCV (3, 4). This has nowadays, however, not resulted in inclusion of cigarette smoking as an important cause of macrocytosis in textbooks and guidelines. Hence, in the current study, we aimed to investigate the association between smoking, assessed by both questionnaire and 24hour urinary cotinine excretion, as objective measurement of nicotine exposure, with MCV in 2 large populationbased cohorts.

First, we analyzed data from the Lifelines cohort study. Lifelines is a large multidisciplinary prospective populationbased cohort study which examines, in a unique 3generation design, the health and healthrelated behaviors of persons living in the north of The Netherlands. For the present study, we included 131886 of the 167729 subjects (aged 1893 years) of whom hematology indices, drinking and smoking behavior were available. Second, we analyzed data from the Prevention of Renal and Vascular EndStage Disease (PREVEND) study, a prospective, population based cohort of Dutch men and women aged 2875 years. For current analyses, we used data from the second survey (N=6894) and excluded missing data on smoking behavior (N=86), resulting in 6808 participants eligible for analyses. Smoking status was categorized as never, former, and current (<6, 620, or >20 cigarettes/d). To exclude possible misclassification or under or overestimation of number of cigarettes smoked per day as determined by questionnaire, 24hour urinary cotinine levels were measured. Alcohol use was categorized as no alcohol use, 1 U of alcohol per month to 1 U/wk, >1 U/wk to 7 U of alcohol per week, >1 U/d to 3 U of alcohol per day, or > 3 U of alcohol per day. Details of the Lifelines cohort and PREVEND study regarding clinical examination, biochemical measurements, data description and statistical analyses are described in the Supporting Information. Similarly, baseline demographics and clinical characteristics of the included 131886 communitydwelling participants and 6808 PREVEND participants are shown in Supplementary Tables 1 and 2.

Of the 131886 Lifelines participants (age 45±13 years, 40% males), 47% were nonsmokers, 33% were former smokers and 20% were current smokers. Of the current smokers, 28% smoked <6 cigarettes per day, 55% smoked 620 cigarettes per day and 18% smoked >20 cigarettes per day. Hemoglobin levels were higher in current smokers (14.3±1.2 g/dL) compared with nonsmokers (14.0±1.3 g/dL, P<0.001). Similarly, MCV levels were higher in current smokers (91.4±4.3 fL) compared with nonsmokers (89.2±4.0 fL, P<0.001, Figure 1A). Macrocytosis was present in 494 (1.9%) of current smokers compared with 166 (0.3%) of nonsmokers (P<0.001, Figure 1B).

In univariable linear regression analysis, current smoking, compared with nonsmoking, was positively associated with MCV (β=0.24, P<0.001). In multivariable regression analysis, performed in the whole cohort, current smoking compared with nonsmoking, remained positively associated with MCV (β=0.23, P<0.001), independent of adjustment for age, sex, estimated glomerular filtration rate (eGFR), body mass index (BMI), and alcohol use. Multivariable regression analysis was also performed in a subgroup of participants from whom also gamma glutamyltransferase (GGT), alanine aminotransferase (ALAT), free thyroxine (FT4), and highsensitivity Creactive protein (hsCRP) were available (N=36109) with the same result (β =0.23, P<0.001).

Similarly, in logistic regression, smoking was a strong determinant of macrocytosis (OR 6.25, 95% CI 5.27.51; P<0.001 in the total cohort, OR 6.00, 95% CI 4.128.73; P<0.001 in the subgroup of N=36109), independent of adjustment for potential confounders.

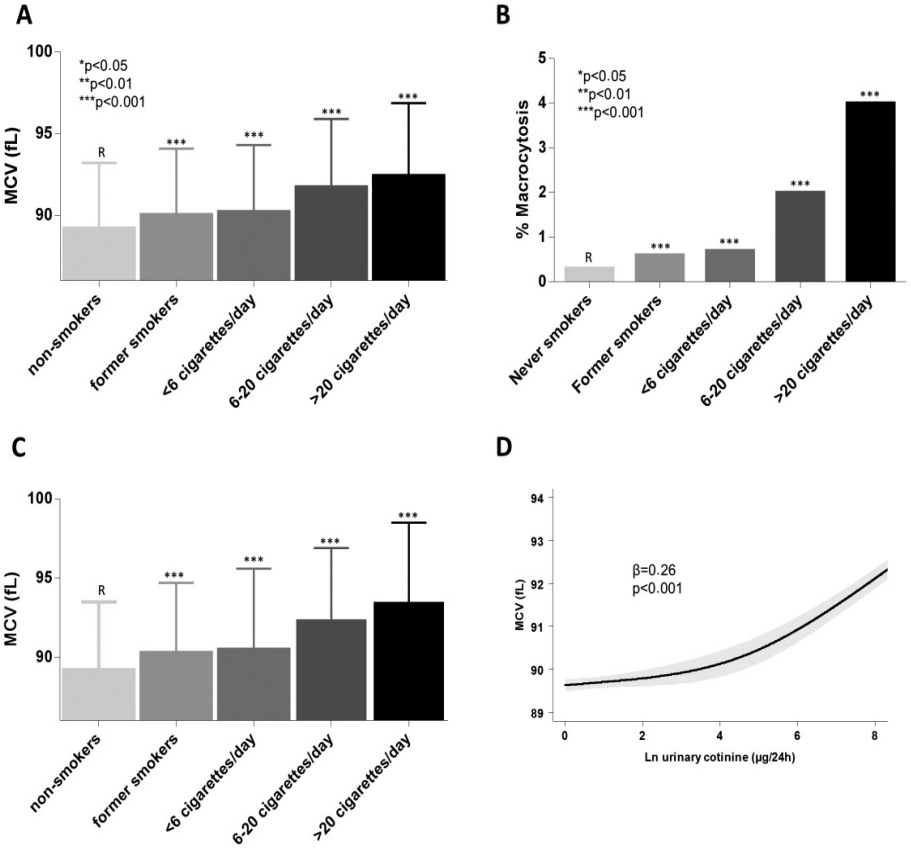

Figure 1. Association of smoking and 24hour urinary cotinine excretion levels with mean corpuscular volume and macrocytosis. A, The association between smoking status and MCV in the lifelines cohort. Reported Pvalues are shown in respect to reference category of nonsmokers. B, The prevalence of macrocytosis for each smoking status in the lifelines cohort. Reported Pvalues are shown in respect to reference category of nonsmokers. C, The association between smoking status and MCV in the PREVEND study. Reported Pvalues are shown in respect to reference category of nonsmokers. D, The association between 24hour urinary cotinine excretion levels and MCV by means of restricted cubic splines. Three knots have been specified at the 10th, 50th, and 90th of 24hour urinary cotinine percentiles. The 95% CIs are indicated by the shaded areas. Twentyfour urinary cotinine levels have been natural log transformed. Abbreviations: MCV, mean corpuscular volume; PREVEND, prevention of renal and vascular endstage disease. *P<0.05 **P<0.01 ***P<0.001

Hereafter, we divided current smoking into categories of cigarettes smoked per day. In multivariate analysis, all smoking categories (<6 cigarettes [β=0.07, P<0.01], 620 cigarettes [β=0.22, P<0.001], and >20 cigarettes [β=0.19, P<0.001]) were associated with MCV, independent of adjustment for potential confounders. The association remained the same after adjustment for GGT, ALAT, FT4, and hsCRP (<6 cigarettes [β = 0.06, P<0.001), 620 cigarettes [β = 0.22, P<0.001], and >20 cigarettes (β=0.21, P<0.001]).

Of the 6808 subjects (age 53±12 years, 50% males) in the PREVEND study, 29% were nonsmokers, 43% were former smokers, and 28% were current smokers. Of the latter, 16% smoked <6 cigarettes per day, 70% smoked 620 cigarettes per day, and 14% smoked >20 cigarettes per day. Hemoglobin levels were higher in current smokers (13.9±1.2 g/dL) compared with nonsmokers (13.6±1.3 g/dL, P<0.001). Similarly, MCV levels were higher in current smokers (92.3±4.7 fL) compared with nonsmokers (89.2±4.3 fL, P<0.001, Figure 1C). Macrocytosis was present in 73 (4%) of current smokers compared with 8 (0.4%) of nonsmokers (P<0.001).

In univariable linear regression analysis, current smoking, compared with nonsmoking, was positively associated with MCV (β=0.30, P<0.001). In multivariable analysis, current smoking, compared with nonsmoking, remained positively associated with MCV (β=0.24, P<0.001), independent of adjustment for age, sex, eGFR, BMI, hsCRP, alcohol use, GGT, ALAT, FT4, vitamin B12, and folic acid. Similarly, in logistic regression, smoking was a strong determinant of macrocytosis (OR, 8.54, 95% CI 2.5728.37; P<0.001), independent of adjustment for potential confounders.

Hereafter, we divided current smoking into categories of cigarettes smoked per day. In multivariate analysis, smoking <6 cigarettes (β=0.03, P=0.06), was not associated with MCV, whereas smoking 620 cigarettes (β=0.24, P<0.001), and smoking >20 cigarettes per day (β=0.13, P<0.001) remained, compared with nonsmoking, associated with MCV, independent of adjustment for potential confounders.

As sensitivity analysis, we repeated in the PREVEND study the analysis with 24 hour urinary cotinine excretion levels as objective reflection of smoking. Twentyfour hour urinary cotinine excretion was strongly correlated with current smoking (β=0.82, P<0.001). Similar to the primary analysis, we identified a strong positive association between 24hour urinary cotinine excretion and MCV (β=0.26, P<0.001, Figure 1D). The association remained independent of adjustment for potential confounders (β=0.23, P<0.001).

In this study, we have shown that smoking, assessed both by means of a selfadministered questionnaire and by 24hour urinary cotinine excretion levels, was strongly positively associated with MCV. Importantly, this association was independent of known causes of macrocytosis, including alcohol use. A few years ago, McNamee et al. (5) and O'Reilly et al. (6) reinvestigated the association between smoking as unrecognized cause of macrocytosis and showed that cigarette smoking was a significant risk factor for macrocytosis, independent of other known causes. Unfortunately, at present cigarette smoking is still not

mentioned in textbooks and major guidelines, and clinicians are generally unaware of this association. The major drawback of the previously performed studies was that smoking status was assessed by means of a selfadministered questionnaire, which might still be regarded as a subjective measurement of smoking status. In this study, we underline the importance of this association, and we are the first to utilize an objective measurement that is, urinary cotinine excretion levels, for the current association. The latter combined with the large patient populations can be regarded also as the major strength of this study. Due to the observational design of this study, we cannot discern potential mechanisms for the strong association between smoking and MCV. Finally, despite the extensive number of factors for which we adjust, residual confounding can still not be excluded.

In conclusion, smoking is an important determinant of MCV levels and macrocytosis, independent of prominent causes such as alcohol intake, liver disease, vitamin B12, and folic acid deficiency. Smoking should be included in current guidelines regarding known causes of an elevated MCV, and the current study might draw more attention to the mechanism by which smoking causes macrocytosis independent of alcohol intake.

Conflict of interest

Nothing to report.

Author contributions

All authors read and approved the final version of the manuscript. M.F.E., H.J.C.M.W., G.H. and S.J.L.B. contributed to the study design. M.F.E. and H.J.C.M.W performed the statistical analysis. M.F.E., H.J.C.M.W., L.M.K., M.M.v.d.K., P.v.d.M., B.H.R.W., C.A.J.M.G., J.E.KR., D.J.T., G.H. and S.J.L.B. contributed to the interpretation of the data and analysis. M.F.E. and H.J.C.M.W. wrote the first draft and all authors edited the paper.

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Supplemental Methods

Study design

Lifelines cohort

Lifelines is a large multi-disciplinary prospective population-based cohort study which examines, in a unique three-generation design, the health and health-related behaviors of persons living in the north of The Netherlands. It employs a broad range of investigative procedures in assessing the biomedical, socio-demographic, behavioral, physical, and psychological factors which contribute to the health and disease of the general population, with special focus on multi-morbidity and complex genetics (1-3). All included participants were between 18 and 93 years old at the time of enrolment between 2006 and 2013. They provided written informed consent before participating in the study. The study protocol was approved by the medical ethical review committee of the University Medical Center Groningen. For the present study, we included 131886 of the 167729 subjects of whom hematology indices, drinking and smoking behavior were available.

PREVEND study

Prevention of Renal and Vascular End-Stage Disease (PREVEND) study is a prospective, population-based cohort of Dutch men and women aged 28-75 years (4). In total, 8592 subjects constituted the PREVEND study at baseline. Details of this cohort have been described elsewhere (5-7). For current analysis, we used data from the second survey (N=6894) and excluded missing data on smoking behavior (N=86), resulting in 6808 participants eligible for analysis. The study has been approved by the local Medical Ethics Committee and written informed consent was obtained from all participants.

Clinical parameters

Lifelines cohort

At baseline, participants completed a self-administered questionnaire on demographics and drinking and smoking behavior. Nonsmokers were participants who had not smoked during the last month and who never smoked longer than one year. Former smokers were participants who smoked during a whole year, but not during the last month. Current smokers were participants who smoked longer than a year and had not stopped smoking. Current smokers were subdivided in categories of number of cigarettes smoked per day, i.e. less than six, six to 20, or

more than 20 cigarettes. Alcohol intake was based on the response to specific questions regarding frequency of alcohol consumption and the average number of units of alcohol per day. Individuals who reported not having consumed alcohol during the past month were considered non-drinkers. The number of alcoholic drinks per week was determined by multiplying the number of drinking days per week by the average number of units consumed on a drinking day. Body weight was measured without shoes to the nearest 0.1 kg. Height was measured to the nearest 0.5 cm. Body mass index (BMI) was calculated by dividing weight in kilograms by the squared height in meters (kg/m2). The specific methodology employed by Lifelines has been described previously (8-10).

PREVEND study

All participants completed a self-administered questionnaire regarding demographics, cardiovascular and renal disease history, smoking habits, alcohol consumption, and medication use. Smoking status was categorized as never, former, and current (less than six, six to 20, or more than 20 cigarettes/day). Alcohol use was categorized as no alcohol use, one unit of alcohol per month to one unit per week, more than one unit per week to seven units of alcohol per week, more than one unit per day to three units of alcohol per day, or more than three units of alcohol per day. After removal of shoes and heavy clothing, weight was measured to the nearest 0.5 kg. Height was measured to the nearest 0.5 cm. BMI was calculated using the same formula as in the Lifelines cohort.

Laboratory parameters

In the Lifelines cohort, hemoglobin, hematocrit, number of erythrocytes and MCV were measured using routine procedures on a XE2100-system (Sysmex, Kobe, Japan). Alanine aminotransferase (ALAT) (IFCC assay, with pyridoxal phosphate activation), gamma-glutamyltransferase (GGT) and creatinine (enzymatic) were routinely measured on a Roche Modular P Platform (Roche, Mannheim, Germany). Free thyroxine (FT4) was assayed by electro-chemiluminescent immunoassay on the Roche Modular E170 analyzer (Roche). High-sensitivity C-reactive protein (hs-CRP) was analyzed using nephelometry (BN II system; Siemens, Marburg, Germany). In the PREVEND study, hemoglobin, hematocrit and MCV were measured using a Coulter Counter STKS sum (Coulter Corporation, Miami, Florida, USA). Hs-CRP was analyzed using nephelometry (BN II system; Siemens). Vitamin B12 and folic acid were measured using an immunoassay based on electrochemiluminesce (Elecsys, Roche). Liver function tests were measured by standardized enzymatic method on a P Modular (Roche). Free T4 was measured by microparticle enzyme immunoassay (AxSYM; Abbott Laboratories, Abbott Park, IL, USA). Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula was applied in both studies

to calculate the estimated glomerular filtration rate (eGFR) (11). Twenty-four hour urinary cotinine levels were measured with Enzyme Multiplied Immunoassay Technique on the Abbott Architect c8000 system (*Abbott* Laboratories, *Abbott* Park, IL). Macrocytosis was defined as a MCV≥100 fL.

Statistical analysis

Data were analyzed using IBM SPSS software, version 23.0 (SPSS Inc., Chicago, IL) and R version 3.2.3 (Vienna, Austria). We evaluated between-group differences using one way ANOVA, Kruskal-Wallis test, or Chi-square test, as appropriate. Hereafter, we performed linear regression analysis between smoking and MCV with adjustment for potential confounders including age, sex, eGFR, body mass index (BMI), hs-CRP levels, categories of alcohol use, ALAT, GGT, FT4 levels, vitamin B12 levels, and folic acid levels in the PREVEND population, and as far as available in the Lifelines population. Furthermore, we performed logistic regression analyses for association between smoking and macrocytosis. We repeated the analyses for categories of number of cigarettes smoked per day. Finally, we measured in all 24-hour urine samples of the PREVEND study urinary cotinine concentrations, to provide an objective and quantitative measure of nicotine exposure. Therefore, to exclude possible misclassification or under- or overestimation of number of cigarettes smoked per day as determined by questionnaire, we repeated as sensitivity analyses the analyses with 24-hour urinary cotinine levels. All reported coefficients are standardized and in all analyses, a two-sided significance level ≤0.05 is being used.

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Supplementary Table 1. Baseline characteristics according to nonsmokers, former smokers, and smokers, with the latter subdivided in amount of cigarettes per day – Lifelines cohort.

			Current smokers			
Variables	**Nonsmokers**	**Former smokers**	**<6 cigarettes/ day**	**6-20 cigarettes/ day**	**>20 cigarettes/ day**	**P value**
	(N=62453)	(N=44005)	(N=7067)	(N=13895)	(N=4466)	
MCV (fL)	89.2 (4.0)	90.0 (4.1)	90.2 (4.1)	91.7 (4.2)	92.4 (4.4)	<0.001
Macrocytosis* (yes, %)	166 (0.3)	263 (0.6)	53 (0.7)	278 (2)	163 (4)	<0.001
General characteristics						
Age (yr)	42 (13)	50 (12)	39 (12)	41 (12)	43 (10)	<0.001
Male sex (n, %)	23596 (38)	18228 (41)	2673 (38)	5575 (40)	2214 (50)	<0.001
BMI (kg/m^2)	25.7 (4.4)	26.6 (4.2)	25.3 (4.0)	25.6 (4.2)	26.5 (4.8)	<0.001
eGFR† (ml/min/1.73m^2)	97.7 (15.0)	92.5 (14.6)	101.3 (14.7)	101.2 (14.4)	101.3 (13.1)	<0.001
Alcohol intake						<0.001
No alcohol use (n, %)	16285 (26)	7539 (17)	815 (12)	2737 (20)	1000 (22)	
1-4 units/month (n, %)	8889 (14)	4103 (9)	494 (7)	1080 (8)	305 (7)	
1-7 units/week (n, %)	27530 (44)	19364 (44)	3319 (47)	5409 (39)	1302 (29)	
≥1-3 units/day (n, %)	8759 (14)	11528 (26)	2133 (30)	3832 (28)	1285 (29)	
>3 units/day (n, %)	990 (2)	1471 (3)	306 (4)	837 (6)	574 (13)	
Laboratory parameters						
Hemoglobin (g/dL)	14.0 (1.3)	14.1 (1.2)	14.1 (1.2)	14.3 (1.2)	14.7 (1.2)	<0.001
hs-CRP (mg/L) ǂ	1.1 (0.7–2.7)	1.2 (0.6–2.6)	1.2 (0.7–3.4)	1.4 (0.7–3.4)	2.0 (0.9–4.5)	<0.001
GGT (U/L) §	24 (14–27)	21 (15–31)	20 (15–28)	21 (16–31)	24 (17–38)	<0.001
ALAT (U/L) ||	19 (14–26)	20 (15–28)	18 (13–26)	18 (13–25)	19 (14–27)	<0.001
FT4 (pmol/L) ¶	15.7 (2.2)	15.7 (2.3)	15.8 (2.2)	16.2 (2.3)	16.5 (2.4)	<0.001

One way ANOVA was applied for determining P values for normally distributed data, Kruskal-Wallis test for P values in skewed distributed data, and Chi-square test for categorical data. P values were calculated across the different groups. Normally distributed data are written as mean (SD), skewed distributed data as median (interquartile range), and categorical data as numbers (percentage). Abbreviations: ALAT, alanine aminotransferase; BMI, body mass index; eGFR, estimated glomerular filtration rate; FT4, free thyroxine; GGT; gamma-glutamyltransferase; hs-CRP, high-sensitivity C-reactive protein; MCV, mean corpuscular volume. * Macrocytosis was defined as a mean corpuscular volume of 100 fL or more.† eGFR was calculated with the CKD-EPI equation. ǂ Available of 42638 participants § Available of 48079 participants. || Available of 48080 participants. ¶ Available of 38842 participants.

Supplementary Table 2. Baseline characteristics according to nonsmokers, former smokers, and smokers, with the latter subdivided in amount of cigarettes per day – PREVEND study.

			Current smokers			
Variables	**Nonsmokers**	**Former smokers**	**<6 cigarettes/ day**	**6-20 cigarettes/ day**	**>20 cigarettes/ day**	**P value**
	(N=1969)	(N=2922)	(N=307)	(N=1346)	(N=264)	
MCV (fL)	89.2 (4.3)	90.2 (4.4)	90.5 (5.1)	92.4 (4.5)	93.5 (5.0)	<0.001
Macrocytosis* (yes, %)	8 (0.4)	29 (1)	3 (1)	50 (4)	20 (8)	<0.001
General characteristics						
Age (yr)	52 (12)	57 (12)	52 (12)	52 (11)	50 (8)	<0.001
Male sex (n, %)	827 (43)	1604 (55)	132 (44)	670 (50)	131 (50)	<0.001
BMI (kg/m^2)	26.6 (4.4)	27.4 (4.4)	26.1 (4.7)	25.8 (4.0)	26.5 (4.5)	<0.001
eGFR† (ml/min/1.73m^2)	88.0 (16.4)	83.4 (17.0)	86.4 (18.3)	85.4 (15.6)	89.7 (14.1)	<0.001
Alcohol intake						<0.001
No alcohol use (n, %)	628 (32)	647 (22)	64 (21)	337 (25)	65 (25)	
1-4 units/month (n, %)	429 (22)	460 (16)	56 (18)	197 (15)	16 (6)	
1-7 units/week (n, %)	622 (22)	920 (32)	107 (35)	412 (31)	66 (25)	
≥1-3 units/day (n, %)	261 (13)	787 (27)	70 (23)	313 (23)	63 (24)	
>3 units/day (n, %)	29 (2)	108 (4)	10 (3)	87 (7)	54 (21)	
Laboratory parameters						
Hemoglobin (g/dL)	13.6 (1.3)	13.7 (1.2)	13.5 (1.3)	14.0 (1.2)	14.2 (1.2)	<0.001
hs-CRP (mg/L)	1.1 (0.5–2.6)	1.4 (0.7–3.0)	1.1 (0.5–3.1)	1.7 (0.8–3.7)	2.5 (1.1–4.6)	<0.001
Vitamin B12 (ng/mL)	313 (119)	313 (124)	292 (111)	302 (121)	314 (107)	0.01
Folate (ng/mL)	13.5 (9.9–19.0)	13.4 (10.0–19.2)	13.4 (9.3–19.1)	11.7 (8.2–16.1)	13.1 (8.6–18.5)	<0.001
GGT (U/L)	21 (14–34)	25 (17–40)	23 (16–40)	26 (17–42)	32 (19–60)	<0.001
ALAT (U/L)	17 (13–25)	18 (13–25)	16 (12–24)	16 (12–23)	17 (12–23)	<0.001
FT4 (pmol/L)	15.6 (2.3)	15.5 (2.3)	15.7 (2.1)	15.9 (2.5)	15.8 (2.3)	<0.001
Urinary cotinine excretion (µg/24h)	0 (0–0)	0 (0–0)	601 (203–1302)	1991 (1301–2877)	2892 (2045–3773)	<0.001

One way ANOVA was applied for determining P values for normally distributed data, Kruskal-Wallis test for P values in skewed distributed data, and Chi-square test for categorical data. P values were calculated across the different groups. Normally distributed data are written as mean (SD), skewed distributed data as median (interquartile range), and categorical data as numbers (percentage). Abbreviations: ALAT, alanine aminotransferase; BMI, body mass index; eGFR, estimated glomerular filtration rate; FT4, free thyroxine; GGT; gamma-glutamyltransferase; hs-CRP, high-sensitivity C-reactive protein; MCV, mean corpuscular volume; PREVEND, Prevention of Renal and Vascular End-Stage Disease. *Macrocytosis was defined as a mean corpuscular volume of 100 fL or more. † eGFR was calculated with the CKD-EPI equation