Free Radical Research, July 2009; 43(7): 677–684

informa
healthcare

SOD3 R231G polymorphism associated with coronary artery disease and myocardial infarction. The Ludwigshafen Risk and Cardiovascular Health (LURIC) study

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(Received 1 April 2009; revised 23 April 2009)

Abstract

This study examined the superoxide dismutase 3 (SOD3) R231G polymorphism in relation to the severity of coronary artery disease (CAD) and the risk of myocardial infarction (MI) in 3211 individuals; 94.4% of study participants were homozygous for SOD3 231RR and 5.5% were heterozygous for SOD3 231RG. The odds ratios of the RG and GG genotype (adjusted for age, gender and for conventional cardiovascular risk factors) were 2.02 (95% CI, 1.23–3.33, $p = 0.005$) for the highest vs the lowest Friesinger coronary score and 1.40 (95% CI, 1.02–1.92, $p = 0.037$) for MI, respectively. Further the SOD3 RG and GG genotype was associated with lower alpha-tocopherol levels than the wild type SOD3 RR genotype. It is concluded that the SOD3 231RG and GG genotype is associated with lower alpha-tocopherol levels and the severity of CAD and the risk of MI.

Keywords: *Coronary artery disease, myocardial infarction, superoxide dismutase, atherosclerosis, polymorphism, alpha-tocopherol*

Introduction

Superoxide dismutase 3 (SOD3, extracellular SOD) is an important anti-oxidative enzyme; it scavenges superoxide anions on the endothelial cell surface of the arterial wall as well as in extracellular fluids [1]. A distinguishing property of SOD3 is its affinity to glycosaminoglycans such as heparin and heparan sulphate [2]. The latter is a component of the glycocalyx of cell surfaces and the connective tissue matrix and appears to be the most important physiological ligand of SOD3 [3,4]. In fact, intravenous injections of heparin lead to an immediate increase in plasma SOD3 [5], allowing the determination of endothelium-bound SOD3 in humans *in vivo* [6].

A single nucleotide polymorphism (SNP) in the SOD3 mRNA at position 691 (C > G) results in a glycine (G) for arginine (R) amino acid exchange at position 231 (R231G, NCBI SNP ID: rs1799895). The glycine for arginine exchange is related to an increased plasma concentration of SOD3 [7–9]. It has been suggested that this increase is due to accelerated release of SOD3 from the interstitial

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ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760902991763

matrix [7]. Several subsequent studies show that the SOD3 231G variant results in an increased concentration of SOD3 in the plasma which is caused by a reduction in the affinities to both heparin and collagen. It appears that the mutation alters the secondary structure in the c-terminal region which mediates the binding to both heparin/heparan sulphate and type I collagen [10]. Thus, despite high plasma concentrations, the arterial wall may be deficient in SOD, leading to diminished scavenging of superoxide anions. The *in vitro* binding studies were extended by a rat model, in which human SOD3 was delivered with a recombinant adenovirus [11]. Constructs containing functionally normal SOD3 and the binding-defective variant were injected into spontaneously hypertensive rats. In contrast to the functionally normal SOD3, the SOD3 231G variant had no significant protective effect on arterial pressure, vascular function or oxidative stress in spontaneously hypertensive rats. Further, wild type SOD3 reduced the concentration of superoxide anion and improved endothelial function in mice, while the SOD3 variant 231G had small effects. These findings suggested that, in contrast to the wild type SOD3, the common human gene variant SOD3 231G fails to protect against endothelial dysfunction.

Insufficient anti-oxidative capacity may promote atherosclerosis through the oxidation of LDL and the formation of peroxynitrite from nitric oxide. It has been reported that SOD3 can protect NO from degradation by superoxide anions generated extracellularly [12] and, on the other hand, NO released from the endothelium stimulates SOD3 expression as well as its release from intracellular pools [10].

Further, high SOD3 levels in plasma have been associated with cardiovascular risk factors [13], acute coronary syndromes [14], hypertension, diabetes mellitus [15], ischemia-reperfusion injury, lung disease, various inflammatory conditions and neurological diseases [12].

All these experimental tests and animal models have provided the mechanistic basis for our association study in which we examined whether humans carrying the SOD3 231RG variant would be predisposed to a greater degree of oxidative stress, consecutively higher degradation of anti-oxidative compounds (e.g. alpha-tocopherol) and a larger burden of atherosclerosis in the coronary arteries leading to myocardial infarction.

Methods

Study design and participants

The *Lu*dwigshafen *Ri*sk and *C*ardiovascular Health (LURIC) study includes consecutive white patients hospitalized for coronary angiography between June 1997 and May 2001. The study was approved by the ethics review committee at the 'Landesärztekammer Rheinland-Pfalz' (Mainz, Germany) and written informed consent was obtained from the participants. A detailed description of LURIC has been published [16].

Diabetes mellitus was diagnosed according to the criteria of the American Diabetes Association (ADA). Further, individuals with a history of diabetes or treatment with oral anti-diabetics or insulin were considered as subjects with diabetes [17].

CAD was assessed angiographically using the maximal luminal narrowing estimated by visual analysis. The severity of CAD was quantified with the Friesinger Score [18]. Previous MI was diagnosed if there was a conclusive positive history of MI or if patients presented with ST elevation or non-ST elevation with typical symptoms of chest pain and/or typical elevations of enzymes (CK, CKMB, troponin T above 0.1 μg/L).

Blood pressure measurements were recorded in the supine position after at least 5 min of rest in the early morning. Patients refrained from smoking or caffeine ingestion before measurement. Systolic and diastolic blood pressures were calculated as the average of at least three measurements taken at 1 min intervals with an automated device. Hypertension was defined as a systolic and/or diastolic blood pressure exceeding 140 and/or 90 mm Hg or a history of hypertension documented by the medical records. Dyslipidemia was defined as HDL cholesterol (HDL-C) levels < 40 mg/dl or LDL cholesterol (LDL-C) levels > 160 mg/dl or triglycerides > 200 mg/dl.

Laboratory procedures

Fasting blood samples were obtained by venipuncture in the early morning after an overnight fast. Plasma glucose, HDL-C, LDL-C, triglycerides were measured by standard laboratory procedures, as described previously [16]. Plasma concentrations of alpha tocopherol were determined by reversed-phase HPLC, as previously described [19]. The within-day coefficient of variation was 2.3%.

Analysis of the SOD3 R231G polymorphism

Genomic DNA was prepared from EDTA anticoagulated peripheral blood by using a common salting-out procedure. Genotyping was done by a 5′-nuclease assay (TaqMan™). Primer and probe sets were designed and manufactured using Applied Biosystems 'Assay-by-Design' custom service. The PCR reaction was performed in a Primus 96 plus thermal cycler (MWG Biotech AG, Germany) using a total volume of 5 μl containing 2.5 μl SuperHot Master Mix (Bioron GmbH, Germany), 0.125 μl assay-by-design mix (Applied Biosystems, Austria), 0.375 μl H_2O and 2 μl DNA. Reactions were overlaid with 15 μl mineral oil. Cycling parameters were: 1 min at 94°C for primary denaturation, followed

by 45 cycles of 15 s at 92°C and 1 min at 60°C. Fluorescence was measured in a VICTOR fluorescence plate reader (HVD Life Sciences, Austria) using excitation/emission filters of 485 nm/520 nm for FAM-labelled probes (231R allele) and 530 nm/572 nm for VIC-labelled probes (231G allele). The data were exported into an Excel format and analysed as scatter plots. As a quality control, genotyping was repeated in 190 samples, showing a discrepant result in one sample which was due to a typing error at the stage of capturing the results. Consequently, genotype data of all samples in the database were compared with raw data, but no further discrepancies were observed. The R231G polymorphism has previously been described as R213G [20–22], but this annotation does not conform with the recommendations for human gene mutation nomenclature [23] and will not be used in this manuscript.

Statistical analysis

Triglycerides were transformed logarithmically prior to being used in parametric statistical procedures. The Friesinger score was broken down to four categories of severity of CAD (0–1, 2–4, 5–8 and 9–15, respectively). Three thousand and thirty-two subjects (94.4%) had the 231RR genotype, 178 (5.5%) had the 231RG genotype and only one individual had the 231GG genotype. Hence, only two groups of SOD genotypes were formed, one including persons with the 231RR genotype and the other one including persons with either the 231RG or the 231GG genotype. The clinical and biochemical characteristics of the study participants according to the Friesinger score strata and to the SOD3 genotypes were expressed as percentages in the case of categorical variables and as means (SD) or medians with inter-quartile ranges in the case of continuous variables (Table I). Continuous variables were compared between groups by univariate analysis of variance. Associations between categorical variables were examined by logistic regression analysis (Table I). The association between the SOD3 genotypes, the Friesinger score and with previous myocardial infarction were studied by logistic regression using the covariables indicated, the SOD3 genotype as the dependent and either the Friesinger score categories or previous MI as the independent variable (Table II).

The concentrations of tocopherols were normalized to LDL cholesterol and multivariate ANOVA models were used to examine the relationships of the Friesinger Score categories with alpha-tocopherol to cholesterol ratios (Table III).

All statistical tests were 2-sided. The criterion for statistical significance was a p-value of less than 0.05. The SPSS statistical package (SPSS Inc., Chicago, IL, version 16.0 for Windows) was used for all analyses.

Results

The SOD3 R231G genotypes were determined in 3211 subjects. The individuals were classified into four groups with increasing severity of coronary artery disease (Friesinger Score 0–1, 2–4, 5–8, 9–15). There were 645, 722, 1034 and 762 individuals in the lowest, second, third and highest category of the Friesinger score, respectively (Table I). As expected, patients with increasing Friesinger score were older, had higher blood pressure, triglycerides and fasting blood glucose, they had lower levels of HDL-C and LDL-C (due to the more frequent use of lipid lowering drugs), had a higher prevalence of diabetes and were more often former and current smokers or male individuals. The body mass index was lower in the lowest and highest category of Friesinger Score compared with the two middle categories.

Three thousand and thirty-two subjects (94.4%) had the 231RR genotype, 178 (5.5%) had the 231RG genotype and one individual had the 231GG genotype. The SOD3 R231G genotypes were in Hardy-Weinberg equilibrium ($p = 0.58$). SOD3 genotype frequencies did not differ between genders. Body mass index, smoking status, LDL-C, HDL-C, triglycerides, age, the prevalence of hypertension and diabetes mellitus did not differ between wild-type individuals (SOD3 231RR genotype) and the mutation carriers (SOD3 231RG and 231GG genotype) (Table I).

The SOD3 231G allele was significantly associated with the severity of CAD. The odds ratio for possessing at least one SOD3 231G allele was 1.18 (95% confidence interval, CI: 1.03–1.37) per increase of the Friesinger score by one category (Table II). This association remained stable after adjustment for age and gender and for traditional cardiovascular risk factors (body mass index, smoking status, hypertension, dyslipidemia and diabetes mellitus). The unadjusted odds ratio for at least one SOD3 231G allele in the Friesinger score category 9–15 vs category 0–1 was 1.63 (95% CI, 1.03–2.57), which was also stable after adjustment for age and gender (1.93, 95% CI, 1.19–3.13) and after multifactorial adjustment for cardiovascular risk factors (2.02, 95% CI, 1.23–3.33).

The unadjusted odds ratio for the presence of at least one SOD3 231G allele was 1.31 (95% CI, 0.97–1.77) among persons with a history of MI. This changed to 1.36 (95% CI, 1.00–1.85) after adjustment for age and gender and to 1.40 (95% CI, 1.02–1.92, $p = 0.037$) after additionally adjusting for other cardiovascular risk factors.

Furthermore, we analysed the relationship between the alpha-tocopherol to cholesterol ratio and the SOD3 polymorphism. SOD3 mutation carriers had significantly lower alpha-tocopherol to cholesterol ratios than persons homozygous for the wild-type. The unadjusted odds ratio for carrying an SOD3

Table I. Clinical and biochemical characteristics of study participants according to Friesinger score and SOD3 R231G genotype.

	Friesinger score					SOD3 231 genotype		
	0–1	2–4	5–8	9–15		RR	RG or GG	p^a
n	645	722	1034	762		3032	179	
Age (years), means ± SD	58 ± 12	64 ± 10	64 ± 10	65 ± 10	<0.001	63 ± 11	62 ± 12	0.264
Male sex (%)	52	64	75	83	<0.001[b]	70	68	0.467[b]
Body mass index (kg/m^2), means ± SD	27 ± 4	28 ± 4	28 ± 4	27 ± 4	0.013	27 ± 4	27 ± 4	0.945
Diabetes mellitus (%)	17	30	35	42	<0.001	32	31	0.972
Systemic Hypertension (%)	63	74	76	77	0.001	73	74	0.569
Smoking								
Never (%)	52	40	30	26		36	40	
Past (%)	31	40	49	55	<0.001	45	38	0.766
Current (%)	18	20	20	20	<0.001	20	22	0.973
Systolic blood pressure (mm Hg), means ± SD	135 ± 22	143 ± 23	143 ± 24	141 ± 24	0.00[c]	141 ± 24	139 ± 23	0.314[c]
Diastolic blood pressure (mm Hg), means ± SD	80 ± 11	82 ± 11	82 ± 11	79 ± 12	<0.001[c]	81 ± 11	80 ± 11	0.163[c]
Fasting blood glucose (g/l), means ± SD	1.04 ± 0.28	1.12 ± 0.34	1.15 ± 0.34	1.19 ± 0.41	<0.001	1.13 ± 0.36	1.13 ± 0.30	0.970
LDL-C(g/l), means ± SD	1.19 ± 0.31	1.19 ± 0.33	1.15 ± 0.37	1.12 ± 0.33	0.002[d]	1.16 ± 0.34	1.19 ± 0.34	0.148[d]
HDL-C (g/l), means ± SD	0.43 ± 0.12	0.40 ± 0.11	0.38 ± 0.10	0.36 ± 0.09	<0.001[d]	0.39 ± 0.11	0.38 ± 0.11	0.425[d]
Triglycerides (g/l) median (25th and 75th percentile)	1.32 (0.98–1.90)	1.50 (1.10–2.06)	1.50 (1.15–1.98)	1.53 (1.13–2.04)	<0.001[d, e]	1.47 (1.09–2.02)	1.38 (1.05–1.92)	0.163[d,e]

[a]Analysis of variance or logistic regression, respectively, adjusted for age and gender; [b]Logistic regression, adjusted for age only; [c]Adjusted for use of beta blockers, ACE inhibitors, AT1 receptor antagonists, calcium channel blockers, diuretics and lipid-lowering agents; [d]Adjusted for use of lipid-lowering agents; [e]ANOVA of logarithmically transformed values.

Table II. SOD3 R231G genotypes, severity of coronary disease and previous myocardial infarction.

	SOD3 231 genotype		Logistic regression					
	RR	RG or GG	Model 1		Model 2		Model 3	
	n (%)	*n* (%)	OR (95% CI)	*p*	OR (95% CI)	*p*	OR (95% CI)	*p*
Friesinger score[a]								
0–1	662 (22%)	31 (17%)	1.0[reference]		1.0[reference]		1.0[reference]	
2–4	687 (23%)	35 (20%)	1.09 (0.66–1.79)	0.739	1.21 (0.73–2.00)	0.467	1.23 (0.74–2.04)	0.432
5–8	975 (32%)	59 (33%)	1.29 (0.83–2.02)	0.260	1.48 (0.93–2.35)	0.100	1.53 (0.95–2.45)	0.80
9–15	708 (23%)	54 (30%)	1.63 (1.03–2.57)	0.035	1.93 (1.19–3.13)	0.008	2.02 (1.23–3.33)	0.005
Per stratum[b]			1.18 (1.03–1.37)	0.022	1.25 (1.07–1.45)	0.005	1.27 (1.08–1.48)	0.003
Myocardial infarction								
No	1758 (58%)	93 (52%)	1.0[reference]		1.0[reference]		1.0[reference]	
Yes	1273 (42%)	87 (48%)	1.31 (0.97–1.77)	0.084	1.36 (1.00–1.85)	0.050	1.40 (1.02–1.92)	0.037

[a]Friesinger score treated as a categorical variable and compared to the reference category. [b]Strata of the Friesinger score treated as interval-scaled variable. Model 1: unadjusted. Model 2: adjusted for age and gender. Model 3: in addition adjusted for type 2 diabetes, body mass index, smoking, hypertension, dyslipidemia.

Table III. SOD3 R231G genotypes and quartiles of the alpha-tocopherol to cholesterol ratio.

	SOD3 231 genotype		Logistic regression					
	RR	RG or GG	Model 1		Model 2		Model 3	
	n (%)	*n* (%)	OR (95% CI)	*p*	OR (95% CI)	*p*	OR (95% CI)	*p*
Alpha tocopherol/cholesterol ratio[a]	2744 (100)	170 (100)						
1. quartile (<5.22)	678 (25)	53 (31)	1.0[reference]		1.0[reference]		1.0[reference]	
2. quartile (5.22–5.96)	671 (24)	52 (31)	0.99 (0.67–1.48)	0.966	1.00 (0.67–1.49)	0.997	1.01 (0.67–1.50)	0.975
3. quartile (5.97–6.92)	699 (26)	31 (18)	0.57 (0.36–0.89)	0.015	0.57 (0.36–0.91)	0.017	0.57 (0.36–0.90)	0.016
4. quartile (>6.92)	696 (25)	34 (20)	0.63 (0.40–0.97)	0.038	0.64 (0.41–1.00)	0.050	0.64 (0.40–1.00)	0.049
Per quartile[b]			0.82 (0.71–0.95)	0.006	0.83 (0.72–0.95)	0.008	0.82 (0.71–0.95)	0.008

[a]Alpha tocopherol/cholesterol ratio treated as a categorical variable and compared to the reference category. [b]Strata of the Alpha tocopherol/cholesterol ratio treated as interval-scaled variable. Model 1: unadjusted. Model 2: adjusted for age and gender. Model 3: in addition adjusted for type 2 diabetes, body mass index, smoking, hypertension, dyslipidemia.

231G allele in the highest vs the lowest alpha-tocopherol to cholesterol quartile was 0.63 (95% CI, 0.40–0.97) and this remained significant after adjustment for age, gender and for cardiovascular risk factors (0.64, 95% CI, 0.40–1.00).

Discussion

The major finding of the present study was that the SOD3 R231G genotype was associated with the severity of CAD and previous MI in persons undergoing angiography. Furthermore, the alpha-tocopherol to cholesterol ratios were significantly lower in carriers of the SOD3 R231G variant.

The SOD3 R231G genotype frequencies correspond to previous reports in which 231RG heterozygotes constituted between 2–6% of the study populations [13,20,22,24,25]. It has been argued that heterozygosity for SOD3 R231G results in lower affinity of the enzyme to heparan sulphate proteoglycans in the glycocalyx of endothelial cell surfaces and in the interstitial matrix of arteries. Petersen et al. [26] have additionally shown that the affinity to collagen in the adventitia of vessel walls is reduced in SOD3 231G carriers. Therefore a reduced local anti-oxidative capacity at the site of the endothelium, the arterial intima or of the adventitia could result in a pro atherogenic micro-environment in the entire arterial wall and consecutively promote atherogenesis.

These experimental findings are supported by a spontaneous hypertensive rat model in which the wild type human SOD3 was shown to be able to protect the endothelium against oxidative damage, whereas the SOD3 231G variant did not [27]. Furthermore in a mice model the SOD 231G variant failed to protect the vessels against endothelial dysfunction induced by an inflammatory stimulus (lipopolysaccharide endotoxin injection) [11].

Thus, an increased prevalence of CAD and MI would be expected in heterozygous carriers of SOD3 R231G. Corresponding findings have been reported from the Copenhagen City Heart Study which has been the first study to demonstrate that SOD3 R231G heterozygosity is associated with an increased risk of CAD in the general population. In their prospective case-control study of 9188 persons the gender- and age-adjusted odds ratio for CAD in heterozygotes vs non-carriers was 1.4 (95% CI 1.0–2.1) and remained stable after multifactorial adjustment. In that study, SOD3 R231G heterozygosity even was more predictive of CAD than the measurement of SOD3 plasma levels [22]. Yamada et al. [28] conducted a study in a highly selected population of haemodialysis patients with type 2 diabetes. The 5-year survival rate of SOD3 R231G heterozygotes was significantly lower than that of non-carriers. Among those who died, the incidence of CAD and cerebrovascular disease in heterozygote carriers was significantly higher than in non-carriers.

Our results are in line with these previous findings and they provide an important extension because the current study is the first one to demonstrate a relationship between the prevalence of SOD3 231G allele and the extent of angiographically ascertained coronary atherosclerosis (Friesinger Score).

Interestingly, in the current study the SOD3 genotype had no influence on serum levels of CRP, fibrinogen, leucocytes, neopterin, oxidized LDL, interleukin 6, CD40 ligand and serum amyloid A (data not shown). This is in line with the concept that the SOD3 R231G polymorphism results in a local depletion of the enzyme SOD at the site of the endothelium which does not translate into changes in systemic indicators of inflammation. Therefore, the pro-oxidative and pro-inflammatory effect of the mutation appears to be a very local process tied to the arterial wall. Hence, we are convinced that the SOD3 genotype contributes in the main independently from other inflammatory markers to the risk of CAD and MI and thus it might be an advantage to measure the SOD3 genotype to assess cardiovascular risk. Many clinically relevant factors influence intermediate biochemical phenotypes. In the case of the SOD3 R231G polymorphism, the relationship to SOD3 concentrations in the circulation is not straight forward. High SOD3 concentrations might reflect high rates of production of the enzyme which would in general protect from oxidative stress. On the other hand, presence of the SOD3 231G genotype might produce high SOD3 concentrations as a consequence of a depletion of SOD3 from the endothelial lineage. In this case, high SOD3 levels could also indicate increased susceptibility to oxidative stress in the arterial wall. These considerations show that the SOD3 R231G polymorphism may provide superior clinical information than serum SOD3 concentrations.

Finally we also found an association between the SOD3 genotype and the antioxidant compound alpha-tocopherol, the main lipid-soluble antioxidant present in lipoproteins [29,30]; which acts as a peroxyl and alkoxyl radical scavenger in lipid environments and thus prevents lipid peroxidation in lipoproteins and cell membranes, for instance of the endothelial cells [31,32]. We suggest that in carriers of an SOD3 231G allele the decreased antioxidant capacity of the endothelium results in an increased consumption of alpha-tocopherol to prevent lipid oxidation. This would then enhance the degradation of alpha-tocopherol in the vessel wall and in lipoprotein particles, lead to lower plasma levels and contribute to the progression of atherosclerosis.

It is in line with the idea that alpha-tocopherol might link the SOD3 231G mutant to atherosclerosis and that alpha-tocopherol exerts a multitude of anti-atherosclerotic effects in experimental and animal studies: arteries deficient in alpha-tocopherol demonstrate a dose-dependent impairment of NO-mediated arterial relaxation upon exposure to ox-LDL [33]; alpha-tocopherol lowers biomarkers of oxidative stress (i.e. F_2-isoprostanes and LDL oxidative susceptibility) [34] and inflammation [35,36], inhibits platelet aggregation [37–39] and monocytes from alpha-tocopherol-supplemented patients have a decreased ability to oxidize lipids or to adhere to an activated endothelial cell monolayer [40].

It is, however, obvious that alpha-tocopherol might not be the sole link between the SOD3 231G variant and atherosclerosis, because most of the vitamin E supplementation trials with cardiovascular endpoints were negative, perhaps due to absence of increased oxidative stress in the individuals or too low intake of alpha-tocopherol [41]. It has been shown that vitamin E supplementation is more effective in special groups of individuals with a higher burden of oxidative stress and high cardiovascular risk [42,43]. Thus, theoretically, SOD3 231G carriers might receive a greater benefit than wild-type individuals.

A limitation of our study is its cross-sectional design. Only individuals in whom coronary angiography was clinically indicated were enrolled in the study which may result in referral bias. On the other hand it is a strength of our study that the coronary status was assessed by angiography in both case and control patients. Furthermore, established cardiovascular risk factors were evaluated accurately which allowed us to control for confounding effects. Moreover, the large sample size of the LURIC cohort provides sufficient proof to detect subtle effects of genetic predictors. Judgement of single nucleotide polymorphisms usually requires several studies to confirm or refute an observed association with the disease of interest [44–46].

In conclusion, the present study confirms the crucial role of superoxide dismutase in arteriosclerosis which was extensively investigated in *in vitro* studies and animal models. So far, very scarce data are available in the light of a clinical setting. We were able to confirm that SOD3 231G allele is significantly associated with the severity of CAD, with previous MI as well as with low levels of alpha-tocopherol.

Acknowledgements

We would like to thank Ernestine Marx-Neuhold and Gerda Grünbacher for their excellent technical assistance, as well as the LURIC study team, the laboratory staff at the Ludwigshafen General Hospital, the University of Freiburg and Ulm University, Germany.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on iFirst on 3 June 2009.