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Circulating Wnt inhibitory factor 1 levels are associated with development of cardiovascular disease

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ABSTRACT

Background and aims: Wnt signaling is involved in atherosclerotic plaque formation directly and indirectly by modulating cardiovascular risk factors. We investigated whether circulating concentrations of Wnt inhibitors are associated with cardiovascular events in subjects with intermediate cardiovascular risk.

Methods: 904 non-diabetic subjects participating in the SAPHIR study were assessed. In the SAPHIR study, middle-aged women without overt atherosclerotic disease at study entry were followed up for 10 years. 88 patients of our study cohort developed cardiovascular disease at follow-up (CVD group). Subjects of the CVD group were 1:2 case-control matched for age, sex, BMI and smoking behavior with subjects without overt cardiovascular disease after a 10 year-follow-up (control group). 18 patients of the CVD group and 19 subjects of the control group were retrospectively excluded due to fulfilling exclusion criteria. Baseline circulating sclerostin, dickkopf (DKK)-1, secreted frizzled-related protein (SFRP)-1 and Wnt inhibitory factor (WIF)-1 levels were assessed by ELISA.

Results: Baseline systemic SFRP-1 and WIF-1 levels were significantly higher in patients with cardiovascular events (n = 70) when compared to healthy controls (n = 157) while DKK-1 and sclerostin levels were similar in both groups. Logistic regression analysis revealed WIF-1 as a significant predictor of future cardiovascular events.

Conclusions: Our data suggest that increased SFRP-1 and WIF-1 levels precede the development of symptomatic atherosclerotic disease. Assessment of systemic WIF-1 levels, which turned out to be independently associated with CVD, might help to early identify patients at intermediate cardiovascular risk.

1. Introduction

Wnt signaling is critically involved in normal cellular processes such as proliferation and differentiation [1–3]. The Wnt family comprises a large group of 19 different proteins [4], which exert their effects via different classes of receptors. By binding to Frizzled (Fz) receptors and co-receptors such as LRP5/6 or receptor tyrosine kinase Ror2/Ryk, Wnt proteins lead to activation of either the canonical (Wnt/β-catenin) or the non-canonical pathway. While canonical Wnt signaling is involved in cell proliferation and fate by

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stabilization of cytosolic β-catenin and subsequent entry into the nucleus, activation of the non-canonical pathway results in release of intracellular Ca^{2+} and as a consequence influences several cellular processes such as cell movement, proliferation and migration [5,6].

Several preclinical and clinical studies suggest that Wnt signaling is involved in pathophysiology of atherosclerosis [7].

Wnt signaling also affects several cardiovascular risk factors: mutations in LDL receptor-related protein (LRP) 6, a co-receptor for soluble Wnt proteins, are associated with hypercholesterolemia, diabetes, hypertension and premature coronary artery disease [12], beyond the suggested direct effects of Wnt signaling on atherogenesis and atherothrombosis, influencing vascular calcification [8–10] and affecting adhesion of monocytes to endothelial cells and proliferation of vascular smooth muscle cells [8,11]. Wnt signaling is critically involved in differentiation and morphogenesis of adipocytes suggesting an indirect role in development of subclinical CVD in obese subjects [13,14].

The aim of our study was to determine whether the circulating Wnt inhibitors sclerostin, Dickkopf-1 (DKK-1), Wnt inhibitory factor 1 (WIF-1) and secreted frizzled related protein 1 (SFRP-1) serve as prognostic biomarkers of future cardiovascular events. Sclerostin is primarily secreted by osteocytes and inhibits the canonical Wnt pathway by binding to LRP 4, 5 and 6 [15]. In addition to its well-known regulatory effects on bone mineralization, sclerostin has been detected in calcified plaques, suggesting a role in vascular calcification [16–18]. DKK-1 is strongly expressed in platelets and adipocytes [19,20] and antagonizes the canonical pathway of Wnt signaling by inhibiting the interaction of Wnt proteins with LRP5/6. Furthermore, DKK-1 has been reported to be involved in inflammatory and angiogenic processes and cell differentiation [20–22]. In contrast to DKK-1 and sclerostin, WIF-1 and SFRP-1 bind directly to Wnt proteins and thereby modulate their ability to form the Wnt receptor complex [8]. SFRP-1 overexpression in bone marrow derived cells was associated with reduced neutrophil infiltration and reduced post-infarction scar in a murine ischemia model [23].

CVD rank among the most important causes of death [24], therefore a robust evaluation of cardiovascular risk is of major importance. Established cardiovascular risk factors such as diabetes, hyperlipidemia and hypertension loom large, but there are still a lot of patients that are not included in this classical risk stratification, who nevertheless are at intermediate risk and need intensive preventive medical care. Therefore, establishment of novel risk factors who identify patients at intermediate risk and at an early stage of disease are of particular importance.

2. Materials and methods

2.1. SAPHIR study cohort

In total 1770 subjects, women aged between 50 and 70 years and men aged between 40 and 60 years were included in the SAPHIR study (Salzburger Atherosklerose Präventionsprogramm bei Personen mit hohem Infarktrisiko) [25]. In this population-based prospective study, patients with history of cerebrovascular, coronary or peripheral artery disease, congestive heart failure, valvular heart disease, chronic alcohol or drug abuse and any chronic disease of the liver or kidney, autoimmune disorders, malignant cancer, hematologic disorders, endocrinopathies, diabetes mellitus or morbid obesity were excluded. Baseline evaluation was performed from 1999-2002 and included measurements of blood pressure, body weight, BMI, analysis of renal and liver function, determination of lipids, estimation of insulin sensitivity using the short insulin tolerance test and the HOMA index as described elsewhere [26], respectively.

Participants were followed up for 10 years. After observation time, medical results and therapies as well as smoking behavior were assessed from medical reports, ICD-9 or 10 diagnosis codes or from patients' questionnaires. Information on cardiovascular death or history of cardiovascular disease defined as myocardial infarction or acute coronary syndrome, coronary angioplasty or bypass surgery, stroke, angioplasty, stenting or bypass surgery for peripheral artery disease, carotid endarterectomy or carotid artery stenting were available for all patients.

2.2. Selection of study subjects

In our study, subjects with full follow-up data and additional specimen available were evaluated for further analysis. Subjects with suspected familial hypercholesterinemia, hypertriglyceridemia (baseline TG > 200 mg/dl) or uncontrolled hypertension at baseline (systolic RR > 160 mm Hg, diastolic RR > 90 mm Hg) as well as subjects who developed any malignancy or diabetes during the 10 year follow up period were excluded from our analysis. 904 out of 1770 subjects fulfilled listed criteria as shown in Fig. 1. 88 patients out of 904 included subjects died from cardiovascular death or had a history of cardiovascular disease after the 10 year follow-up period (CVD group). Patients of the CVD group were 1: 2 case-control matched for age, sex, BMI and smoking behavior (current smoker/non-smoker) with subjects who did not develop any symptomatic cardiovascular disease during the observational period (control group). Retrospectively, 18 patients from the CVD group and 19 subjects from the control group were excluded from analysis due to elevated fasting glucose levels (≥126 mg/dl) or LDL-cholesterol levels > 190 mg/dl determined by further laboratory measurements. In total, 70 patients of the CVD group and 157 subjects of the control group were included for further analysis. 34 subjects took antihypertensive drugs at baseline and 12 subjects were on lipid lowering agents at baseline for primary prevention only.

2.3. Laboratory measurements

Serum and plasma samples were obtained during baseline visit after a fasting period of 8 h. Specimens were immediately processed, frozen and stored at -80 °C until further evaluation. Anthropometric data were determined as described in detail elsewhere [26]. Circulating levels of Wnt inhibitors were determined using commercially available kits from R&D Systems (human Sclerostin and human DKK-1), MYBioSource (human WIF-1) or Cusabio (human SFRP-1). Analyses were performed according to the manufacturer's instructions.

2.4. Common carotid artery intima media thickness (CCA-IMT) measurement

In a subgroup of subjects (CVD group: n = 67 and control group: n = 153) CCA-IMT measurements were performed. Measurements were performed at baseline as described in detail elsewhere using a ATL HDI 3000 CV (Philips Medical Systems, Bothell, WA, USA) [27].

2.5. Statistical analysis

Descriptive data are expressed as mean and standard deviation (SD) in case of normal distribution and as median and interquartile range [IQR] in case of non-normal distribution. Normality of data was tested by Shapiro-Wilk test. In case of non-normal distribution data were log-transformed to try to achieve normal distribution. In case of non-normal distribution of log transformed data, differences between the groups were analyzed by non-parametric tests,

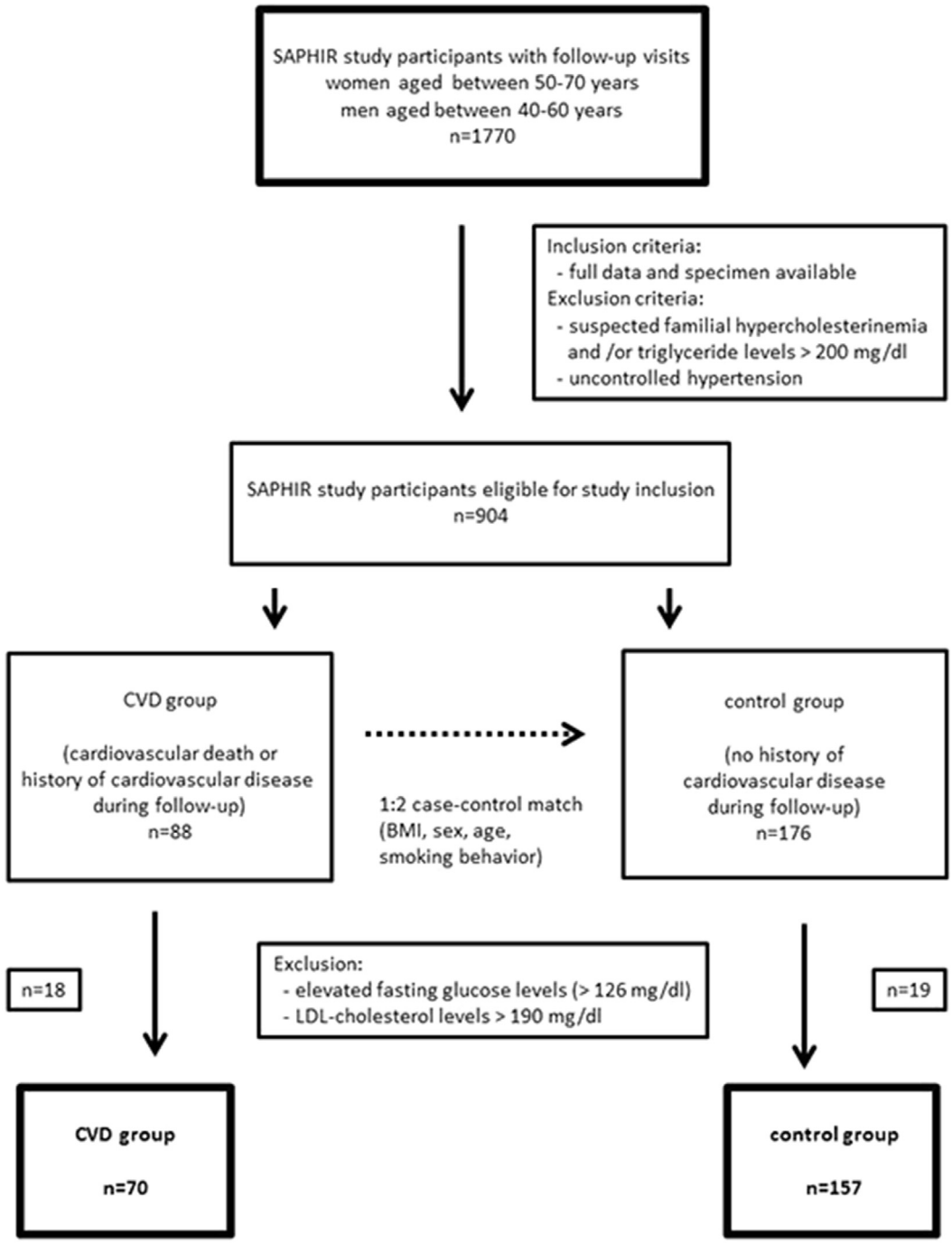

Fig. 1. Study design and selection of participants.

normal distributed data were analyzed by parametric tests. Differences in non-normal distributed circulating levels of sclerostin, DKK-1, SFRP-1, WIF-1 and laboratory measurements between the CVD and the control group and sex specific analysis in men as well as CCA-IMT measurements were evaluated by non-parametric Mann-Whitney U test. Differences in any normally distributed laboratory values between female CVD patients and healthy controls were tested by unpaired t-test. Associations between parameters were estimated by bivariate correlation analysis and were expressed as Pearson correlation coefficient. In order to evaluate the predictive value of Wnt inhibitor concentrations for development of CVD, the Odds Ratio (OR) was estimated by logistic regression analysis. Besides classical cardiovascular risk factors (BMI, age, sex, LDL-cholesterol, fasting glucose) Wnt-inhibitor concentrations that differed significantly between the CVD and the control group at a p-value <0.1 were included in regression analysis. Diagnostic ability of significant predictors of CVD as determined by logistic regression analysis was estimated by ROC analysis. ROCs were compared by the method of DeLong [28]. Unless otherwise specified statistical significance was inferred at a two-tailed p value of less than 0.05 and 95% confidence intervals were used. SPSS for Windows (23.0) and MedCalc (v17.9) was used for statistical analysis.

3. Results

3.1. Subjects characteristics

In total, 227 subjects, 70 patients with overt cardiovascular disease (CVD group) and 157 without overt cardiovascular disease (control group) were evaluated. In our study the median follow-up time was 4.0 years [4.0–5.0]. Baseline characteristics of analyzed subjects are shown in Table 1. Expectedly, LDL-cholesterol as well as triglyceride levels were significantly higher in patients with CVD when compared to healthy controls. HDL-cholesterol, BMI, fasting glucose, HbA1c and HOMA-index were comparable in both groups (see Table 2).

In the CVD group, 19 patients suffered from an ischemic stroke and 25 patients from an acute coronary syndrome during follow-up, 9 were diagnosed with peripheral artery disease, 7 patients underwent carotid artery stenting or endarterectomy, 29 patients underwent coronary angioplasty and stent implantation and 4 patients underwent coronary artery bypass grafting, respectively.

3.2. Wnt inhibitor levels

While DKK-1 and sclerostin levels (DKK-1: CVD group: 2082.4 pg/ml [1558.1–2500.4] *vs.* control group: 2123.5 pg/ml [1825.7–2674.2], $p = 0.1$; sclerostin: CVD group: 92.7 pg/ml [68.7–124.0] *vs.* control group: 97.4 pg/ml [77.1–131.3], $p = 0.5$) did not differ between the groups, SFRP-1 and WIF-1 concentrations were significantly higher in the CVD group than in the control group (SFRP-1: CVD group: 3221.8 pg/ml [2352.8–3811.0] *vs.* control group: 2609.0 pg/ml [1926.1–3234.9], $p = 0.005$; WIF-1: CVD group: 275.1 pg/ml [207.2–335.4] *vs.* control group: (232.9 pg/ml [171.4–303.3], $p = 0.004$) (Fig. 2).

When sex-specific analysis was performed, SFRP-1 and WIF-1 levels were significantly higher in male patients with CVD than in male healthy controls (SFRP-1: CVD group: 3050.6 pg/ml [2157.8–3689.6] *vs.* control group: 2535.4 pg/ml [1760.3–3040.3], $p = 0.006$), WIF-1: CVD group: 282.9 pg/ml [208.0–357.69] *vs.* control group: 232.8 pg/ml [177.0–302.4], $p = 0.003$). When female study participants were analyzed separately, SFRP-1 and WIF-1 levels were comparable in the CVD and the control group probably due to small sample size (SFRP-1: CVD group: 3500.83 ± 919.46 pg/ml *vs.* control group: 3612.12 ± 1469.42 pg/ml, $p = 0.95$; WIF-1: CVD group: 254.01 ± 67.78 pg/ml vs control group: 234.78 ± 107.40 pg/ml, $p = 0.44$). Circulating SFRP-1 levels were significantly higher in women then in men (women: 3260.5 pg/ml [2706.5–4445.3]; men: 2601.7 pg/ml [1795.4–3248.9], $p < 0.001$).

3.3. Correlation analysis

In contrast to DKK-1, WIF-1 and sclerostin, circulating SFRP-1 concentrations were inversely correlated with lean mass (r = -0.4, $p < 0.001$) and body weight (r = -0.2; $p < 0.001$). SFRP-1 was positively (r = 0.3; $p < 0.001$) and WIF-1 negatively (r = -0.2; $p = 0.001$) correlated with HDL levels at baseline. Additionally, WIF-1 was directly associated with fasting triglyceride levels at baseline (r = 0.2; $p = 0.018$). Correlation analysis between circulating levels of Wnt inhibitors and other established cardiovascular risk factors did not reveal any relevant correlations (data not shown).

3.4. Regression and ROC curve analysis

Well-known cardiovascular risk factors age, BMI, fasting glucose, LDL-cholesterol, sex as well as Wnt inhibitor concentrations that differed significantly at a p-value < 0.1 between the CVD and control group were included in the logistic regression analysis. Circulating WIF-1 levels ($p = 0.003$; OR: 1.6 [1.2–2.2]) and LDL-cholesterol concentrations ($p < 0.001$; OR: 1.2 [1.1–1.4]) were significant predictors of future CV events. When sex specific analysis was performed, the significant association of WIF-1 with CVD was only detectable in males ($p = 0.001$) but not in females ($p = 0.50$). When performing ROC curve analysis we found that area under the curve (AUC) non-significantly increases after adding WIF-1 into the calculation (established CV risk factors AUC 0.72 [0.65–0.8]; established CV risk factors and WIF-1 AUC 0.76 [0.69–0.83]; $p = 0.11$) (Fig. 3).

3.5. Carotid intima media thickness

Additionally, CCA-IMT measurements were available from 220 analyzed subjects. None of the subjects had clinically significant focal plaque formation. Median CCA-IMT in subjects participating in our study was 0.775 mm [0.73-0-88]. We did not find a significant difference between the CVD group and control group (CVD group: 0.80 mm [0.73–0.90] *vs.* control group: 0.78 mm [0.71–0.85]; $p = 0.091$).

4. Discussion

Cardiovascular disease is the most common cause of death in the western world [24]. While many major and minor risk factors have been identified [29], the underlying pathophysiological mechanisms are still under investigation. In the past Wnt signaling was found to be critically involved in atherogenesis by regulating and affecting endothelial inflammation, calcification, and

Table 1
Baseline characteristics of study patients.

	CVD group	Control group	*p* value
Age [y]	55 [50.0–57.0]	52 [48.5–56.0]	0.08
BMI [kg/m^2]	27.4 [24.2–29.7]	26.0 [24.4–28.9]	0.11
Sex	53m/17f	123m/34f	0.66
Smoker/non-smoker	19/51	44/113	0.89
Fasting glucose [mg/dl]	93.0 [86.0–99.0]	91.0 [84.5–97.0]	0.07
HbA1c [%]	5.6 [5.4–5.7]	5.6 [5.4–5.7]	0.56
HOMA [index]	1.7 [0.9–2.5]	1.3 [1.0–2.0]	0.13
LDL-C [mg/dl]	152.2 [123.9–174.9]	134.6 [109.3–152.7]	**<0.001**
Fasting triglycerides [mg/dl]	130.0 [80.0–196.0]	100.0 [78.0–159.0]	**0.03**
HDL-C [mg/dl]	54.0 [42.5–62.0]	54.0 [47.0–65.5]	0.24
ASCVD [10-year risk score]	7.5 [4.7–11.5]	5.2 [2.9–8.5]	**0.001**

Median and interquartile ranges are shown.
BMI: body mass index; HOMA: homeostatic model assessment; LDL-C: low density lipoprotein; TG: triglyceride; HDL-C: high density lipoprotein, ASCVD 10year risk score: atherosclerotic cardiovascular disease 10-year risk score.

Table 2
Predictors of CVD (logistic regression analysis).
(A) Logistic regression analysis including established cardiovascular risk factors and (B) extended analysis including established cardiovascular risk factors and Wnt inhibitors.

Logistic regression model including established risk factors				Logistic regression model including established risk factors and Wnt inhibitors			
(A)	OR	95% CI	*p*-value	(B)	OR	95% CI	*p*-value
Sex	1.57	0.7–3.8	0.31	Sex	1.42	0.6–3.6	0.46
Age [years; 5 units]	1.11	0.8–1.5	0.51	Age [years; 5 units]	1.16	0.8–1.6	0.38
BMI [kg/m^2]	0.98	0.9–1.1	0.63	BMI [kg/m^2]	0.98	0.9–1.1	0.68
Smoker/non-smoker	1.50	0.8–2.8	0.21	Smoker/non-smoker	1.45	0.8–2.8	0.27
Systolic BP [mmHg; 5 units]	1.13	1.1–1.2	<0.001	Systolic BP [mmHg; 5 units]	1.12	1.0–1.2	0.01
LDL-C [mg/dl; 10 units]	1.23	1.1–1.4	<0.001	LDL-C [mg/dl; 10 units]	1.23	1.1–1.4	<0.001
Triglycerides [mg/dl; 10 units]	1.01	1.0–1.0	0.37	Triglycerides [mg/dl; 10 units]	1.01	1.0–1.0	0.61
Fasting glucose [mg/dl; 5 units]	1.11	0.9–1.3	0.26	Fasting glucose [mg/dl; 5 units]	1.10	0.9–1.3	0.33
				DKK-1 [pg/ml]	1.00	1.0–1.0	0.68
				Sclerostin [pg/ml]	1.00	1.0–1.0	0.71
				SFRP-1 [pg/ml]	1.00	1.0–1.0	0.56
				WIF-1 [pg/ml; 100 units]	1.60	1.1–2.1	0.01

Data are shown as odds ratio (OR) and confidence interval (CI). OR was calculated for given concentration categories as shown in squared brackets.

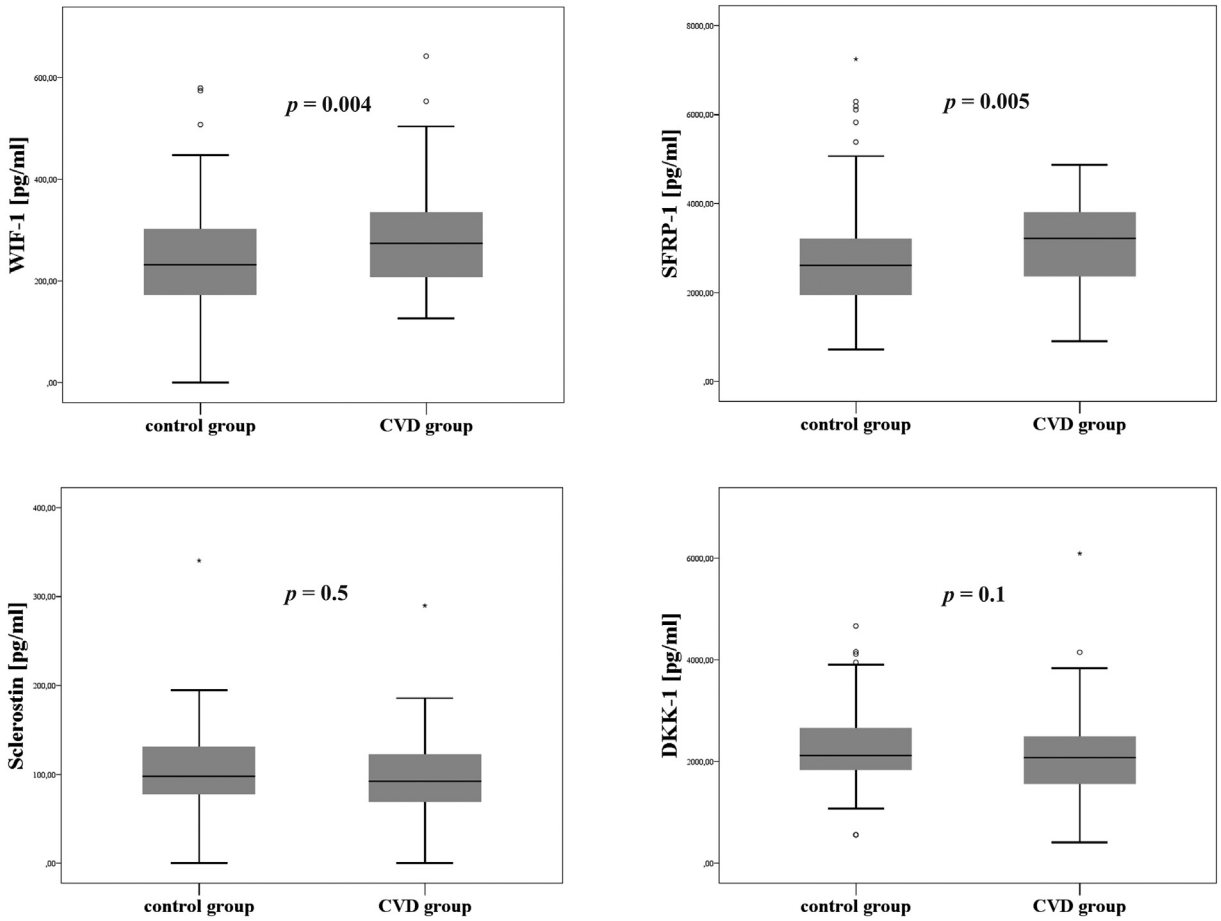

Fig. 2. Baseline circulating WIF-1, SFRP-1, sclerostin and DKK-1 levels in patients of the CVD group and healthy controls.

mesenchymal stem cell differentiation [7,8]. As an example, Wnt5a, which is a member of the Wnt family, is not only implicated in diabetes and metabolic disease but was also found to be associated with the severity of atherosclerotic lesions probably due to its inflammatory functions [30,31]. Additionally, Wnt ligand LRP6 was reported to influence carotid atherosclerotic plaque formation [32].

Wnt signaling activity is influenced by several systemic proteins including sclerostin, DKK-1, WIF-1 and SFRP-1 [33]. While very high cardiovascular risk is very obvious and well-established in patients with familial hypercholesterinemia or type 2 diabetes, more precise characterization of cardiovascular risk is requested for many middle-aged subjects who are categorized as being at moderate to intermediate cardiovascular risk. The latter category is especially addressed in our study by including non-diabetic subjects with an average ASCVD 10-year risk score of 5–10% and only slightly increased age-adjusted intima media thickness [34], without clinically relevant focal plaque formation or any other symptomatic atherosclerotic disease. The aim of this study was to test whether levels of Wnt inhibitors are eligible as markers of future cardiovascular disease and thus might help to early identify subjects who

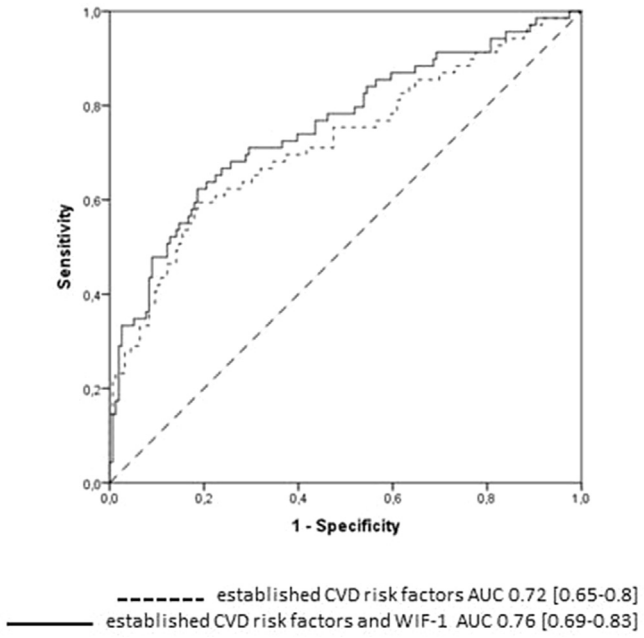

Fig. 3. ROC for established cardiovascular risk factors (sex, age, BMI, smoker/non-smoker, systolic BP, LDL-C, triglycerides, fasting glucose) and improvement of ROC after addition of WIF-1 to the calculation.
The area under the curve (AUC) non-significantly increases after adding WIF-1 into the calculation (established CV risk factors AUC 0.72 [0.65−0.8]; $p < 0.001$; established CV risk factors and WIF-1 AUC 0.76 [0.69−0.83]; $p < 0.001$). The difference between AUC of established risk factors and AUC of established risk factors including WIF-1 did not reach statistical significance ($p = 0.11$).

would benefit from intensive cardiovascular risk reduction. Medical information concerning CV events was obtained from medical reports as well as from patient's questionnaires, which might be a limitation of our study.

In contrast to WIF-1 and SFRP-1, DKK-1 and sclerostin concentrations did not differ significantly in patients who developed cardiovascular disease during the follow-up and healthy subjects. In previous studies DKK-1 levels were associated with coronary artery calcification in patients admitted to hospital with chest pain [35]. Retrospective analysis of patients with acute coronary syndrome showed that high DKK-1 levels were associated with increased rates of major adverse cardiac events during a 2 year follow-up [36]. Seifert-Held et al. [37] reported increased DKK-1 levels in patients with acute ischemic stroke when compared to patients with stable cerebrovascular disease. Additionally, DKK-1 concentrations were higher in patients with stable cerebrovascular disease than in healthy controls. Partly conflicting data might be explained by differences in study designs: while in our work circulating Wnt inhibitor levels were determined many years before manifestation of CVD, DKK-1 levels were evaluated in patients with overt atherosclerotic disease in other studies. While previous studies indicated a role of DKK-1 in clinically symptomatic CVD and plaque destabilization [19,35−37], results from our study population suggest that DKK-1 is not eligible as a marker for future cardiovascular disease in asymptomatic subjects.

Similar to DKK-1 levels, serum sclerostin concentrations were reported to be elevated in diabetic patients with atherosclerotic disease [38] and to be independently associated with carotid intima thickness in type 2 diabetics [39]. In our study including non-diabetic subjects without overt cardiovascular disease at baseline serum sclerostin levels did not serve as a predictor of future cardiovascular disease.

In contrast to DKK-1 and sclerostin, we found that circulating WIF-1 and SFRP-1 levels were significantly higher in study participants who developed CVD during the follow up period. Mechanistically, WIF-1 was found to reduce platelet-derived growth factor-BB (PDGF-BB)-induced vascular smooth muscle cell (VSMC) proliferation which causes intimal thickening in atherosclerosis [40]. SFRP-1 was shown to influence the cell cycle of endothelial cells and smooth muscle cells (SMC) and subsequently reduces cell proliferation and capillary density in ischemic muscles [41]. Additionally, SFRP-1 was found to inhibit WNT signaling via binding to the WNT complex [8]. From our findings, it might be speculated that WIF-1 and SFRP-1 levels are upregulated in early steps of atherosclerosis in order to initiate or strengthen anti-proliferative counter-acting mechanisms. Consequently, WIF-1 and SFRP-1 levels might serve as markers of atherosclerotic activity.

When performing logistic regression analysis, baseline WIF-1 levels turned out to be associated with CVD in addition to LDL-cholesterol concentrations even after controlling for well-known cardiovascular risk factors such as age, sex, BMI and fasting glucose concentrations. Similar results were found when other analyzed Wnt inhibitors that differed between the CVD and control group were included in regression analysis. These results indicate that WIF-1 levels might serve as a marker of future cardiovascular disease. Noteworthy, the case control study design with matched subjects for sex, age, BMI and smoking habit, might lead to an underestimation of classical risk factors in this analysis, which, as a consequence, might limit the power of this finding. Nevertheless, our data suggest WIF-1 as a novel long-term predictor of CVD in non-diabetic subjects with an overall moderate to intermediate cardiovascular risk. From a clinical perspective, these results might help to better and early identify patients categorized as being at moderate to intermediate cardiovascular risk who would benefit best from early and intensive preventive measures. Lack of correlation of WIF-1 levels with IMT might be explained by the selection of study participants: both patients at very high cardiovascular risk, such as patients with familial hypercholesterinemia or type 2 diabetes, and young subjects with very low cardiovascular risk were excluded from our study.

In summary, we report that WIF-1 and SFRP-1 levels are elevated in subjects who developed cardiovascular disease during the follow-up period. Mechanistically, upregulation of WIF-1 and SFRP-1 might indicate a protective counter-regulatory anti-atherosclerotic activity. Circulating WIF-1 turned out to be independently associated with CVD suggesting that assessment of systemic WIF-1 might help to early identify patients who could benefit best from intensive and early cardiovascular preventive measures. These results are restricted to middle-aged subjects with moderate-to intermediate cardiovascular risk.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

CR performed statistical analysis und wrote the manuscript. AS, KR, KS, GS performed laboratory analysis. GG, AT performed statistical analysis. MP, KW, LK, BI investigated study subjects. HT contributed to discussion, BP designed the SAPHIR study. SK designed the study and wrote the manuscript.

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