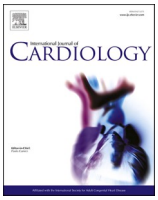
International Journal of Cardiology 406 (2024) 132035

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard



High dose statin treatment reduces circulating Dickkopf-1 following acute myocardial infarction

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ARTICLE INFO

Keywords:
Myocardial infarction
Statins
Dickkopf-1

ABSTRACT

Background: Secreted glycoproteins of the Dickkopf (DKK) family modify Wnt signaling and may influence plaque destabilization but their modulation by statins in MI patients is not known.
Methods: We measured plasma DKK-1 and DKK-3 in patients with acute ST-segment elevation MI (STEMI) before percutaneous coronary intervention (PCI) and after 2 and 7 days and 2 months in patients receiving short-term high-dose (40 mg rosuvastatin, given before PCI; $n = 25$) and moderate dose (20 mg simvastatin, given the day after PCI; $n = 34$). In vitro modulation of DKK-1 in human umbilical vein endothelial cells (HUVECs) by statins were assessed.
Results: (i) Patients receiving high dose rosuvastatin had a marked decline in DKK-1 at day 2 which was maintained throughout the study period. However, a more prevalent use of β-blockers in the simvastatin group, that could have contributed to higher DKK-1 levels in these patients. (ii) There was a strong correlation between baseline DKK-1 levels and change in DKK-1 from baseline to day 2 in patients receiving high dose rosuvastatin treatment. **(iii)** DKK-3 increased at day 2 but returned to baseline levels at 2 months in both treatment groups. (iv) Statin treatment dose-dependently decreased DKK-1 mRNA and protein levels in HUVEC.
Conclusions: Our findings suggest that high dose statin treatment with 40 mg rosuvastatin could persistently down-regulate DKK-1 levels, even at 2 months after the initial event in STEMI patients.

1. Introduction

Whereas Wingless (Wnt) signaling is critically important for developmental processes, reactivation of the Wnt pathways is involved in various cardiovascular diseases such as atherosclerosis and its clinical consequences [1,2]. Secreted glycoproteins of the Dickkopf (DKK) family modulate Wnt signaling and may influence atherosclerotic disease progression [2]. Indeed, DKK-1 is released from platelets and endothelial cells in patients with coronary artery disease, potentially contributing to plaque progression and destabilization [3–5]. In contrast, DKK-3 displays anti-atherogenic effects, attenuates myocardial remodeling and protects against cardiac dysfunction in experimental models of myocardial infarction (MI) [6,7]. These contrasting effects are reflected systemically, where elevated circulating DKK-1 was observed in patients with atherosclerotic disorders [4,8], independently associated with poor prognosis in acute coronary syndromes (ACS) [9,10], while DKK-3 was inversely related to carotid artery intima-media thickness and 5-year progression of carotid atherosclerosis [6]. Several studies suggest statins have anti-atherogenic effects beyond lipid lowering [11], and recently, Pontremoli et al. demonstrated that statins directly attenuate DKK-1 in vascular cells in vitro [12]. Whereas these authors also found that atorvastatin lowered DKK-1 plasma levels in rabbits fed a cholesterol-rich diet [12], there are, to the best of our knowledge, no clinical data on the effect of statins on DKKs in humans

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[1] This author takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

https://doi.org/10.1016/j.ijcard.2024.132035
Received 19 October 2023; Received in revised form 25 March 2024; Accepted 7 April 2024
Available online 9 April 2024

including patients with atherosclerotic disease. In the present study, we therefore evaluated if plasma DKK-1 and DKK-3 were modulated by short-term aggressive (rosuvastatin 40 mg) statin therapy given before percutaneous coronary intervention (PCI) in patients with ST-segment elevation MI (STEMI) followed for 2 months. We also sought to replicate dose-dependent in vitro effects of statins on endothelial cells.

2. Methods

The MICROS study was a prospective single-center trial carried out at Stavanger University Hospital in Norway in the period December 2011 – February 2015. Written informed consent was obtained from all participants and the study was performed in accordance with the Declaration of Helsinki, approved by the Regional ethical committee, Western Norway Health Authority (2010/269), registered in the clinical trial database (Clintrials.gov NCT013824722) (EudraCT number 2010–021796-93). Patients were included if they had (1) no previous MI, (2) demonstrated acute proximal/mid-occluded single vessel disease, (3) underwent successful PCI with stent implantation without significant residual stenosis. Patients with multivessel disease, cardiac arrest, cardiogenic shock, stent thrombosis, previous MI, angina within 48 h before admission, previous coronary artery bypass grafting, atrial fibrillation, pacemaker, concurrent inflammatory, infectious or malignant disease, biliary obstruction or hepatic insufficiency were excluded. No patients should have received intravenous fibrinolysis prior to PCI. Twenty-five consecutive recruited patients with first time STEMI defined by typical chest pain and ST elevation on the electrocardiogram at admission received short-term aggressive statin therapy (i.e., rosuvastatin 40 mg daily [the first dose before PCI] the first week and thereafter simvastatin 40 mg). As a control group we used samples from the KOMPIS study ($n = 34$) (Clintrials.gov: NCT 00465868) [13], a study with identical inclusion and exclusion criteria, where the patients received simvastatin 20 mg throughout the study period (first dose given the following day after PCI). In both studies all patients were treated with aspirin and clopidogrel. Angiotensin-converting enzyme inhibitors/angiotensin receptor blockers were initiated within 36 h of PCI in all patients.

In both studies blood samples were collected immediately before PCI at day 2, 7, and 60. Pyrogen-free blood collection tubes containing tethylenediaminetetraacetic acid were immediately immersed in melting ice and centrifuged within 20 min at 2500*g* for 20 min to obtain platelet-poor plasma. All samples were stored at −80 °C and thawed <3 times. Plasma DKK-1 and DKK-3 were measured by ELISA (RnD systems, Stillwater, MN) with intra- and inter-assay CV% < 10.

Cell culture experiments: Primary human umbilical vein endothelial cells (HUVEC) were obtained from umbilical cord veins by digestion with 0.1% collagenase A (Boehringer Mannheim GmbH, Mannheim, Germany) and cultured to confluence for 2–4 days as previously described [14]. Media was then discarded and HUVEC stimulated with 5 and 20 μM ortho-hydroxy atorvastatin (gift from Pfizer, New York, NY) for 6 and 24 h and cell-free supernatants and cell were harvested and stored at −80 °C. The endotoxin levels of all stimulants and culture media were < 10 pg/mL (Limulus Amebocyte assay; BioWhittaker, Walkersville, MD). For qPCR, total RNA was extracted from HUVECs and platelets using RNeasy columns (Qiagen), subjected to DNase I treatment, and stored in RNA storage solution (Ambion, Austin, TX) at −80 °C. Primers for DKK-1 (forward primer [FP]: 5′- GGGAATTACTGCAAAAATGGAATA-3′ and reverse primer [RP]: 5′- ATGACCGGAGACAAACAGAAC-3′). Gene expression of the housekeeping gene GAPDH (Applied Biosystems) was used for normalization.

Baseline characteristics were compared using unpaired parametric and non-parametric tests depending on distribution of continuous variables while chi-square was used to compare proportions. For analyzing treatment effects between the two statin regimes, DKK-1 and DKK-3 levels were log transformed due to skewed distribution and analyzed with repeated measures ANOVA using baseline levels and a composite score for medications different between the groups as covariates. Data are presented as estimated marginal means and were compared with within- and between-group Sidak adjusted post-hoc tests.

The association between baseline and change in DKK levels was evaluated with Pearson correlation. *P*-values are two-sided and considered significant when <0.05.

3. Results

Patient characteristics in relation to treatment are listed in Table 1 showing comparable groups, but with some difference in medication use. As shown in Fig. 1A, whereas patients receiving 20 mg simvastatin displayed no significant changes during follow-up, patients receiving high dose rosuvastatin had a marked decline in DKK-1 at day two which was maintained throughout the study, with significant within and between group changes at day 2 and 2 months compared to both baseline and patients receiving simvastatin. This was seen also after adjusting for a composite score for ACEi/ARB, β Blocker and aldosterone antagonist use (Fig. 1A). When assessing the influence of medication use that differed between the groups (i.e. ACEi/ARB, β Blocker and aldosterone antagonist) separately, we noticed that β blocker usage (Fig. 1B top) was associated with increased DKK-1 levels towards the end of observation suggesting our findings could partly be due to an enhancing effect of β Blockers in the low dose group. Indeed, adjustment for β Blocker use attenuated the association between statin use and DKK-1 (Fig. 1B, bottom), but a decline in DKK-1 in the high dose rosuvastatin was still observed with a significant difference between groups at 2 days. For DKK-3, no between group differences were observed. However, an increase at day 2, followed by a decrease at day 7 as compared with baseline, before returning to baseline levels at 2 months was observed in both treatment groups (Fig. 1C). Finally, there was a strong correlation between baseline DKK-1 levels and change in DKK-1 from baseline to day 2 in patients receiving high-dose rosuvastatin treatment (Fig. 1D, $r = -0.87$, $p < 0.001$).

To support that statins per se could modulate DKK-1, we assessed the dose-dependent effect of statin (i.e., ortho-hydroxy atorvastatin) treatment on the expression and secretion of DKK-1 in HUVEC endothelial cells. As shown in Fig. 1E and F, statin treatment dose-dependently decreased DKK-1 mRNA expression and protein secretion with the most prominent effects at 6 and 24 h for mRNA and protein,

Table 1
Baseline characteristics of study group recorded immediately before percutaneous coronary intervention (PCI), where applicable ($n = 59$).

	20 mg Simvastatin ($n = 34$)	40 mg rosuvastatin ($n = 25$)	p
Age (years)	58.6 ± 11.8	58.7 ± 10.9	0.92
Male gender (n (%))	28 (82)	21 (88)	0.87
Current smoker n (%)	14 (52)	14 (56)	0.14
Diabetes mellitus n (%)	2 (6)	1 (4)	0.74
Hypertension	8 (24)	6 (25)	0.97
Symptom to reperfusion time (min)	201 [117, 340]	162 [130,202]	0.34
Culprit vessel			
Left anterior descending artery n (%)	16 (47)	15 (60)	0.33
Right coronary artery n (%)	15 (44)	8 (32)	0.35
Circumflex artery n (%)	3 (9)	2 (8)	0.91
Medication n (%)			
Clopidogrel	34 (100)	25 (100)	1.00
Aspirin	34 (100)	25 (100)	1.00
Statin	34 (100)	25 (100)	1.00
ACEi/ARB	25 (74)	24 (96)	0.023
β Blocker	17 (50)	4 (16)	0.009
Aldosterone antagonist	4 (13)	19 (76)	<0.001

TIMI = Thrombolysis In Myocardial Infarction. ACEi/ARB, Angiotensin-converting enzyme inhibitors/angiotensin receptor blockers.

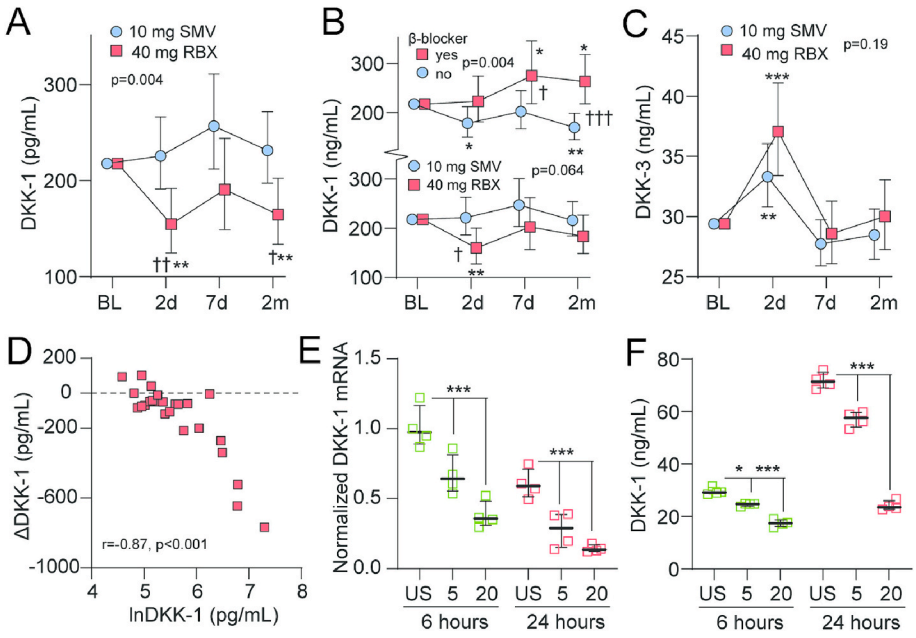

Fig. 1. Time profiles of DKK-1 plasma in 59 patients with ST-segment elevation MI before (baseline) and 2, 7, and 60 days after primary PCI according to **A)** treatment with 10 mg simvastatin (SMV) or 40 mg rosuvastatin (RBX), adjusting for a composite score for ACEi/ARB, β Blocker and aldosterone antagonist use. The top of panel **B)** shows DKK-1 according to β-blocker use while the bottom panel shows DKK-1 according to statin use as in A, adjusting for β-blocker use. **C)** shows DKK-3 according to statin treatment as described for panel A. *$p < 0.05$. **$p < 0.01$, ***$p < 0.001$ vs. baseline. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ comparing the two groups at different time-points. **D)** Correlation (Pearson) between baseline DKK-1 and change in DKK-1 from baseline to 2 days in patients receiving 40 mg rosuvastatin. The effect of ortho-hydroxy atorvastatin (5 and 20 μM) on **E)** DKK-1 mRNA and **F)** protein levels in conditioned media after 6-and 24-h stimulation in HUVECs. $N = 4$ for each condition. *$p < 0.05$, ***$p < 0.001$ vs. unstimulated (US).

respectively.

4. Discussion

Statins have been shown to downregulate DKK-1 in endothelial cells and smooth muscle cells [12] and breast cancer cell lines [15] and suggested as a novel target for statin therapy [16]. However, there are no studies reporting these effects in vivo in humans. The present study supports and extends these previous in vitro and pre-clinical findings showing that atorvastatin down-regulate DKK-1 in endothelial cells at both the mRNA and protein level in a dose-dependent manner. Moreover, the effect of short-term aggressive statin treatment (rosuvastatin 40 mg) initiated before PCI and continued for 1 week, followed by 20 mg simvastatin throughout the study period, as compared to moderate statin treatment (simvastatin 20 mg) given the first day after PCI, was associated with decreased levels of circulating DKK-1 in STEMI patients even two months after inclusion. Intriguingly, a higher proportion of the patients that were treated with simvastatin received β-blockers and our data suggest that the use of this medication could potentially be a confounder in these analyses by enhancing DKK-1 levels, at least partly contributing to the differences between the two statin regimens. To the best of our knowledge, this is the first report of a possible interaction between b-blockers and DKK-1, and this should be further investigated in forthcoming studies including the influence of these widely used medications on Wnt-signaling. Our in vitro data support the findings of Pontremoli et al., who provided evidence that statins directly inhibit DKK-1 production in vascular cells and suggested that a substantial portion of pleiotropic statin effects are mediated through a down regulation of DKK-1 [12]. These include effects on extracellular matrix organization, platelet activation and response to wounding processes, supporting previous observations by us that DKK-1 may enhance inflammatory interactions between platelets and endothelial cells, potentially contributing to atherogenesis and plaque destabilization [4]. Furthermore, we and others have demonstrated that high circulating DKK-1 in patients with ACS and stroke is independently associated with poor prognosis [9,10,17]. Thus, a down-regulatory effect of high dose statin on DKK-1 may be clinically relevant and based on the strong negative correlation between baseline DKK-1 and change in DKK-1 in those receiving high dose statin could be considered a treatment option in high-risk patients presenting with high circulating DKK-1 on admission.

In contrast to DKK-1, and despite a high sequence homology between DKK-1 and DKK-3, DKK3 may potentiate Wnt signaling through interactions with the Kremen co-receptors [18], potentially attenuate myocardial remodeling and protect against cardiac dysfunction following experimental MI [7,19]. If the increase in DKK-3 at 2 days reflects a protective upregulation to limit cardiac remodeling or is related to statin treatment is not known. However, the sharp increase in DKK-3 at 2 days followed by a decrease at 7 days compared to the sustained effect of high-dose statin on DKK-1 may suggest that the upregulation of DKK-3 reflects the natural course of DKK-3 in STEMI.

5. Conclusion

Although some limitations such as different time for initiation high-dose and moderate -dose statin therapy (before and after PCI), some differences in other cardiovascular medications and lack of more direct comparison in a randomized controlled trial, our findings suggest that short-term high dose statin treatment with 40 mg rosuvastatin initiated before PCI could persistently down-regulate DKK-1 levels in first time STEMI patients, even at 2 months after the initial event, as compared with moderate-dose simvastatin, supporting the use of aggressive stain therapy in high risk STEMI patients. However, the more prevalent use of β-blockers in the simvastatin groups may have influenced our data, and the effect of b-blockers on Wnt-signaling should be further investigated.

Sources of funding

The Western Norway Regional Health Authority (ref. 911,697).

Funding

Noreen Butt received funding from the Western Norway Regional Health Authority (ref. 911697).

Author statement

The datasets generated and/or analyzed during the current study are not publicly available due to ethical restrictions from the Regional Committee for Medical and Research Ethics in South-East Norway but are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Thor Ueland: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Noreen Butt:** Writing – review & editing, Investigation, Data curation. **Tove Lekva:** Writing – review & editing, Methodology. **Stein Ørn:** Writing – review & editing, Investigation, Data curation. **Cord Manhenke:** Writing – review & editing, Data curation. **Pål Aukrust:** Writing – review & editing, Writing – original draft, Conceptualization. **Alf Inge Larsen:** Writing – review & editing, Writing – original draft, Project administration, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors report no relationships that could be construed as a conflict of interest.

Acknowledgements

We appreciate the help of study nurse, Jorunn Nilsen, for her participation in the local organization of the trial.

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