Effect of statin therapy on the carboxypeptidase U (CPU, TAFIa, CPB2) system in patients with hyperlipidemia : a proof-of-concept observational study

Effect of statin therapy on the carboxypeptidase U (CPU, TAFIa, CPB2) system in hyperlipidemic patients: a proof-of-concept observational study.

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Abstract

Purpose: Statins are commonly used in patients with hypercholesterolemia to lower cholesterol and reduce their cardiovascular risk. There is also considerable evidence that statins possess a range of cholesterol-independent effects, including profibrinolytic properties. This pilot study aimed to explore the influence of statins on procarboxypeptidase U (proCPU) biology and to search for possible effects and associations that can be followed up in a larger study.

Methods: Blood was collected from sixteen patients with hyperlipidemia, before and after three months of statin therapy (simvastatin 20 mg or atorvastatin 20 mg). Fifteen age-matched normolipemic subjects served as controls. Lipid parameters and markers of inflammation and fibrinolysis (proCPU levels and clot lysis times) were determined in all samples.

Findings: Mean (SD) proCPU levels were significantly higher in patients with hypercholesterolemia compared to control subjects (1186 [189] vs. 1061 [60] U/L). Treatment of these patients with a statin led to a significant average decrease of 11.6% in proCPU levels and brought the proCPU concentrations to the same level as in the control subjects. On a functional level, enhancement in plasma fibrinolytic potential was observed in the statin group, with the largest improvement in fibrinolysis seen in patients with the highest baseline proCPU levels and largest proCPU decrease upon statin treatment.

Implications: Increased proCPU levels are present in patients with hyperlipidemia. Statin treatment significantly decreased proCPU levels and improved plasma fibrinolysis in these patients. Moreover, our study indicates that patients with high baseline proCPU levels are most likely to benefit from statin therapy. The latter should be examined further in a large cohort.

Keywords

- Carboxypeptidase B2
- Carboxypeptidase U
- HMG-CoA reductase inhibitors
- Hypercholesterolemia
- Thrombin-Activatable Fibrinolysis Inhibitor

Main text

Introduction

Statins (3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors) are the cornerstone of lipid-lowering therapy and are widely used in primary and secondary prevention of cardiovascular diseases (CVD).[1,2] Over the years it has been established that the beneficial effects of statins on morbidity and mortality can not solely be attributed to their lipid-lowering properties.[3,4] There is considerable evidence of cholesterol-independent effects (so-called 'pleiotropic effects'), among which profibrinolytic properties (e.g. influence on tissue factor, platelet aggregation and fibrinolysis).[5,6]

The antifibrinolytic enzyme carboxypeptidase U (CPU, TAFIa, CPB2) is present in the circulation as its inactive precursor procarboxypeptidase U (proCPU, TAFI, proCPB2). Upon activation by thrombin(-thrombomodulin) or plasmin, the zymogen is converted into the active enzyme.[7,8] Once activated, the enzyme potently attenuates fibrinolysis by cleaving C-terminal lysines on partially degraded fibrin, thereby interfering with efficient plasminogen activation.[9]

The effect of different statins on circulating proCPU levels has been studied, showing the potential of statins to modulate the CPU system, thereby positively affecting fibrinolysis.[10–14] A number of these studies, however, have methodological drawbacks.

With this proof-of-concept observational study we aimed to explore the influence of statin therapy on proCPU biology in a limited number of statin-treated hypercholesterolemic patients to search for possible effects and associations that can be followed up in a subsequent larger study with the ultimate goal to further elucidate the effect of this treatment on fibrinolysis. Participants' proCPU levels, as well as their plasma fibrinolytic potential, were assessed before and after three months of therapy. Additionally, the potential relationship with lipid parameters and markers of fibrinolysis and inflammation was studied.

Participants and Methods

Study design

Hypercholesterolemic patients (total cholesterol (TC) ≥190 mg/dL or low-density lipoprotein (LDL) cholesterol ≥115 mg/dL) with a 10-year risk of fatal CVD of ≥5% (calculated with the Systematic Coronary Risk Estimation chart) were enrolled and allocated to simvastatin 20 mg or atorvastatin 20 mg by the collaborating general practitioners (GP).[2] Age-matched normolipemic subjects, presenting at the GP for a routine physical exam or renewal of chronic prescription drugs, were recruited as controls. Exclusion criteria for both groups were the clinical history of cardiovascular events, (chronic) liver disease, pregnancy, a glomerular filtration rate <30 mL/min, the use of anticoagulant or lipid-lowering drugs and initiation of a new medication (other than statin therapy) during the course of the study. The study was approved by the local ethics committee (B300201837918) and all participants gave written informed consent before enrolment.

Sample collection

Blood samples were collected in hypercholesterolemic individuals at the time of inclusion (baseline) and after three months of statin therapy. In controls, two blood collections were performed three months apart. Participants' blood was drawn in fasting state between 8 and 10 a.m. Blood intended for proCPU determination, clot lysis experiments, measurement of soluble thrombomodulin (sTM) and high sensitive C-reactive protein (hsCRP) was drawn in 3.2% trisodium citrate tubes (Vacutainer, BD; 9:1 v/v). Serum separator tubes (Vacutainer, BD) were used for the determination of a lipid panel. After collection, one tube of citrated whole blood was kept apart for the isolation of genomic DNA. All other tubes were centrifuged at 2000 *x g* for 15 min at 4°C, aliquoted and stored at -80°C in our biobank (BE71030031000) until further analysis.[15]

ProCPU measurement

Plasma was diluted 40-fold in HEPES (4-2-hydroxyethyl-1-piperazineethaansulphonic acid; 20 mmol/L, pH 7.4; Merck, Germany) whereafter proCPU was activated with human thrombin (Merck, Germany), rabbit-lung thrombomodulin (Seikisui Diagnostics, USA) and $CaCl_2$ (Merck, Germany)(final concentrations 4 nM, 16 nM, and 50 mM respectively). Subsequently, the active CPU was incubated with the in-house substrate Bz-*o*-cyano-Phe-Arg and the formed product quantified by high-performance liquid chromatography.[16]

Clot lysis assay

In vitro clot lysis was assessed by mixing citrated plasma with tPA (tissue plasminogen activator; final concentration 40 ng/mL) alone or tPA in combination with PTCI (potato tuber carboxypeptidase inhibitor; final concentration 75 µg/mL; Merck, Germany). Coagulation was initiated by addition of $CaCl_2$ (final concentration 12.5 mM) and turbidity was measured spectrophotometrically every 30s at 405nm and 37°C (Versamax, Molecular Devices, USA).[17,18] The clot lysis time (CLT; the time between half-maximal turbidity during coagulation and fibrinolysis) and ΔCLT (the absolute reduction in CLT after addition of PTCI) were calculated.[17]

Lipid measurement

TC, high-density lipoprotein (HDL) cholesterol, and triglycerides (TG) were measured using a Siemens Healthineers Atellica™ CH Analyzer (Siemens Healthcare Diagnostics, Canada). LDL-cholesterol was calculated based on the Friedewald formula.

Other measurements

hsCRP and sTM antigen levels were measured by ELISA according to the manufacturer's instructions (Human CRP ELISA Kit and Thrombomodulin Human ELISA kit, Abcam, UK). Oxyhemoglobin levels were determined to exclude the potential influence of hemolysis on the proCPU- and clot lysis assays.[19] Detection of the Thr325Ile proCPU polymorphism was performed according to Zorio *et al*.[20]

Statistical Analysis

Results were expressed as mean ± standard deviation (SD). GraphPad Prism version 8 was used for statistical analysis and data plotting. Continuous variables were evaluated with a Mann-Whitney test (unpaired data) or a Wilcoxon Matched-Pairs Signed Rank test (paired data). A chi-square test was used for categorical data. Spearman correlation coefficients were computed to assess the following associations: ΔproCPU–baseline proCPU levels, ΔproCPU–ΔLDL-cholesterol, ΔproCPU–ΔCRP and baseline ΔCLT–change in ΔCLT. Results with $p<0.05$ were considered statistically significant.

Results

Study population

Sixteen statin-treated hypercholesterolemic patients (participation rate was 89%) and fifteen normolipemic controls were included in the study between November 2018 and May 2020. Baseline characteristics of the study population are summarized in Table 1. Sex ($p=0.04$), systolic blood pressure ($p=0.001$) and the use of antihypertensive and antiaggregant drugs (both $p=0.04$) differed significantly between both groups. All other characteristics were balanced.

Lipid profiles of statin patients and controls are shown in Table 2. At the time of inclusion, TC- and LDL-cholesterol were significantly higher in the statin group (both $p=0.002$). After three months of statin therapy, both parameters reduced significantly (both $p<0.001$) and were even lower compared to the controls (although not statistically significant for TC), pointing towards good therapy adherence.

Statin therapy downregulates proCPU levels

Mean baseline proCPU levels were significantly higher in statin-eligible patients compared to controls (1186±189 vs. 1061±60 U/L; $p=0.04$) (Table 2 and Fig 1A). Statin therapy in these patients led to a significant decrease of 138 U/L (11.6%, $p=0.002$) in plasma proCPU levels after three months compared

to baseline, while proCPU levels remained the same in controls. At the end of the study, proCPU levels did not differ anymore between patients and controls (p=0.18).

A positive correlation was observed between baseline proCPU levels and the decrease in proCPU under influence of statin treatment (ΔproCPU) (r=0.88, p<0.001; Fig 1C). Both baseline proCPU levels and the decrease in proCPU showed high inter-individual variation. Three subpopulations were identified: a cohort with baseline proCPU levels (1063±47 U/L) similar to controls (1061±46 U/L) and no proCPU decrease under influence of a statin (p=0.63); a second cohort with baseline proCPU levels (1104±56 U/L) similar to controls and a 5-10% decrease in proCPU levels after statin use (p=0.06); and a cohort in which baseline proCPU levels were 20% higher (1316±225 U/L) and in which statin treatment resulted in approximately 20% decrease after three months (p=0.02) (Fig 1B).

Downregulation of procarboxypeptidase U by statin therapy has a non-lipid related pleiotropic character

To evaluate the nature of the effect of statins on the CPU system, the correlation between the difference in proCPU levels and the decrease in LDL-cholesterol levels was investigated. No association was observed between these parameters (r=0.26, p=0.32).

Plasma CRP was significantly increased in hyperlipidemic patients (p=0.04) and decreased by statin therapy (48%; p<0.001), while unchanged in the control population (Table 2). No association was found between the statin-induced reduction in both CRP and proCPU levels (r=0.04, p=0.87).

Plasma fibrinolytic potential improves under the influence of statin therapy

Participants' CLTs were measured as an indicator of their plasma fibrinolytic potential. CLTs at inclusion tended to be higher in statin-treated patients compared to controls (68.7±27.4 min vs. 65.4±10.7 min; p=0.34). Furthermore, the CLT did not change in controls during follow-up (mean change +1.4 min; p=0.87), whereas this parameter was significantly lowered in statin-treated patients (mean change -3.2 min; p=0.03) (Table 2).

The addition of the carboxypeptidase inhibitor PTCI resulted in similar CLTs (presented as CLT_{PTCI} in Table 2) in patients and controls at both time points. The ΔCLT (absolute reduction in CLT after addition of PTCI) was shortened by 3.0 min (p=0.001) under influence of statin therapy, while the ΔCLT was not significantly changed over time in controls (+1.2 min; p=0.74) (Table 2 and Fig 1D).

A correlation was observed between the baseline ΔCLT and the change in ΔCLT after three months of statin treatment (r=0.57, p=0.035; Fig 1F). Moreover, the mean change in ΔCLT corresponded with the observed decrease in proCPU levels in the earlier described subpopulations, with the largest improvement in fibrinolysis seen in the subpopulation with the highest baseline proCPU levels and largest proCPU decrease upon statin treatment (Fig 1E and 1G).

sTM levels were the same in controls and statin patients at both the time of inclusion (3.3±0.2 ng/mL and 3.1±1.2 ng/mL respectively; p=0.38) and three months later (3.1±0.4 and 3.0±0.8 respectively; p=0.74; Table 2).

Discussion

The finding of increased proCPU levels in patients with hyperlipidemia (compared to age-matched controls) complies with previously reported results.[10–12] Moreover, a proCPU decrease following statin treatment was also seen before.[10–12] However, this is the first study to identify subpopulations based on the high inter-individual variation observed in both baseline proCPU levels and the decrease in proCPU. At least in the subpopulation in which baseline proCPU levels were 20% higher and in which statin treatment resulted in approximately 20% decrease after three months an improvement in fibrinolysis is to be expected. Based on the association between high proCPU levels and an increased risk of cardiovascular disease, these individuals with high proCPU concentrations are likely to benefit most from statin therapy.[21]

The nature of the effect of the HMG-CoA-reductase inhibitors on the CPU system (lipid- or nonlipid-related) was further investigated. The lack of an association between the difference in proCPU levels and the decrease in LDL-cholesterol levels, suggests that statins downregulate plasma proCPU concentrations in a nonlipid-related manner.

In agreement with previous research, an increase in plasma CRP levels was observed in hyperlipidemic patients as well as a decrease of this parameter under influence of statin treatment.[22] The reduction in CRP during statin therapy is described to be independent of the magnitude of the LDL-cholesterol reduction and is considered indirect proof for the presence of pleiotropic effects of statins.[22,23] In this light, the correlation between the statin-induced reduction in both CRP and proCPU levels was investigated. Since no association was found, the mechanism underlying the reduction of both these parameters seems to be different.

Measurement of the participants' CLTs showed that statin administration improved the fibrinolytic potential in the statin cohort. A profibrinolytic effect of statins was also observed in 126 patients with prior venous thromboembolism receiving rosuvastatin.[12] In the same study, the fibrinolytic potential was reported to be unchanged during follow-up in 121 non-rosuvastatin users.[12]

Moreover, the observation that the CLT_{PTCI} was similar in both groups at both time points is an indication that the *in vitro* fibrinolytic capacity excluding the CPU system was neither significantly affected by hyperlipidemia nor by statin therapy. However, when evaluating the actual contribution of the CPU system to the total clot lysis time (ΔCLT), a mean change over time of -3.0 min was observed for statin patients. Based on the CPU threshold mechanism, the mean reduction of 138 U/L in proCPU levels under influence of statin therapy theoretically corresponds to a reduction in the ΔCLT of approximately 2.7 min.[24] This is in line with the observed change in the ΔCLT.

Furthermore, the fact that the largest improvement in fibrinolysis is seen in those statin-treated patients with the highest baseline proCPU levels and the largest proCPU decrease, indicates that the profibrinolytic effect seen in statin users can – at least partly – be explained by the downregulation of plasma proCPU concentrations. Although the clinical relevance (reduction in morbidity and mortality)

of the observed phenomenon is not yet clear, these observations underline that especially individuals with high proCPU concentrations are likely to benefit from statin therapy.

Traces of sTM prolong fibrinolysis as a result of their ability to increase the catalytic efficiency of proCPU activation 1250-fold.[9] Increased sTM levels can therefore result in prolonged CLTs. In our study sTM levels were equal in controls and statin patients at both time points. This is a strong indicator that the difference in ΔCLT during follow-up is the result of lower proCPU levels and can not be attributed to alterations in proCPU activation.

Our study confirms recently published data and additionally focuses on all details of the proCPU/CPU system and also has a longer follow-up time.[12] Furthermore, particular attention was paid to the selection of the proCPU assay: proCPU measurements were performed with a thoroughly validated activity-based assay that allows selective measurement of proCPU and is not accompanied by important limitations, including unequal reactivity towards different isoforms of the proCPU 325 polymorphism.[16,25,26] Due to the small sample size and the statin and control populations not being sex-matched, results serve as pilot data that need to be explored in a large cohort. In addition, further research is needed to more accurately determine the target proCPU level to obtain a beneficial effect of statin therapy, as well as to unravel whether the proCPU downregulation under influence of statin therapy is a class-mediated effect and if the effect is dose-dependent.

Conclusions

This pilot study shows that increased proCPU levels are present in hyperlipidemic patients. Treatment of these patients with a statin led to a significant average decrease of 11.6% in proCPU levels and brought the proCPU concentrations to the same level as in controls. On a functional level, improvement in plasma fibrinolysis (as measured by the change in ΔCLT) was observed in the statin group, which could be linked to the observed proCPU decrease. Moreover, the largest improvement in fibrinolysis was seen in patients with the highest baseline proCPU levels. The latter should be

examined further in a large cohort not only to identify the clinical relevance of this observation but also as it puts forward the hypothesis that it might be valuable to include proCPU measurement in the risk-assessment for starting statin therapy.

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Figures and Tables

Table 1 – Baseline characteristics of patients eligible for statin therapy and controls

	Controls	Statin patients	*p*-value
Demographics			
Number – N	15	16	
Age – years (range)	66 (53 – 80)	69 (49 – 80)	0.32 [a]
Male sex – N (%)	8 (53)	3 (19)	0.04 [b]
BMI – kg/m² (SD)	24.4 ± 3.6	27.8 ± 5.3	0.09 [a]
Smoking – N (%)	5 (33)	4 (25)	0.62 [b]
Genotype [c]			0.66 [b]
Ile/Ile – N (%)	1 (7)	1 (6)	
Thr/Ile – N (%)	10 (67)	7 (44)	
Thr/Thr – N (%)	4 (27)	8 (50)	
Blood pressure			
Systolic – mmHg (SD)	129 ± 10	141 ± 8	0.001 [a]
Diastolic – mmHg (SD)	79 ± 6	83 ± 7	0.21 [a]
Medication use			
Antihypertensive – N (%)	5 (33)	13 (81)	0.04 [b]
Antiaggregant – N (%)	0 (0)	6 (38)	0.04 [b]
Antidiabetic – N (%)	0 (0)	1 (6)	0.37 [b]
Antidepressant – N (%)	1 (7)	2 (13)	0.71 [b]
Statin therapy			
Simvastatine 20 mg – N (%)	-	5 (31)	-
Atorvastatine 20 mg – N (%)	-	11 (69)	-

Results are given as number (N) with percentage in parentheses or as mean ± standard deviation (SD). A Mann-Whitney U-test [a] or a Chi-square test [b] were used to test for statistical significant between-group differences.

[c] Single nucleotide polymorphism +1040C/T, corresponding to a Thr/Ile substitution at position 325 of proCPU.

Table 2 – Biochemical and hemostatic parameters of statin patients and controls at inclusion (baseline) and after three months

		N	Baseline (mean ± SD)	After three months (mean ± SD)	Mean change	*P*-value [a]
Lipid parameters						
TC (mg/dL)	Statin patients	16	239 ± 37	172 ± 27	-67	<0.001
	Controls	15	188 ± 25	180 ± 23	-8	0.50
	p-value [b]		0.002	0.48		
LDL-C (mg/dL)	Statin patients	16	147 ± 32	82 ± 28	-65	<0.001
	Controls	15	115 ± 29	123 ± 29	+8	0.63
	p-value [b]		0.002	0.01		
HDL-C (mg/dL)	Statin patients	16	66 ± 17	72 ± 14	+6	0.09
	Controls	15	50 ± 7	47 ± 8	-3	0.25
	p-value [b]		0.44	0.002		
TG (mg/dL)	Statin patients	16	135 ± 66	96 ± 40	-39	0.02
	Controls	15	114 ± 16	90 ± 24	-24	0.13
	p-value [b]		0.66	0.83		
Markers of fibrinolysis and inflammation						
ProCPU (U/L)	Statin patients	16	1186 ± 189	1048 ± 124	-138	0.002
	Controls	12	1061 ± 60	1066 ± 44	+5	>0.99
	p-value [b]		0.04	0.18		
CLT (min)	Statin patients	15	68.7 ± 27.4	65.5 ± 23.1	-3.2	0.03
	Controls	11	65.4 ± 10.7	66.8 ± 22.0	+1.4	0.87
	p-value [b]		0.34	0.75		
CLT_{PTCI} (min)	Statin patients	15	42.7 ± 15.9	42.4 ± 11.6	-0.3	0.76
	Controls	11	39.5 ± 8.2	39.7 ± 13.2	+0.2	>0.99
	p-value [b]		0.63	0.95		
ΔCLT (min)	Statin patients	15	26.1 ± 12.4	23.1 ± 14.3	-3.0	0.001
	Controls	11	25.9 ± 13.4	27.1 ± 15.6	+1.2	0.74
	p-value [b]		0.37	0.01		
sTM (ng/mL)	Statin patients	16	3.1 ± 1.2	3.0 ± 0.8	-0.1	0.75
	Controls	12	3.3 ± 0.2	3.1 ± 0.4	-0.2	0.13
	p-value [b]		0.38	0.74		
hsCRP (mg/L)	Statin patients	16	6.3 ± 1.5	3.3 ± 5.4	-0.30	<0.001
	Controls	12	1.9 ± 2.9	2.0 ± 1.0	+0.01	0.81
	p-value [b]		0.04	0.84		

Results are presented as mean ± standard deviation (SD). [a] Wilcoxon Matched-Pairs Signed Rank test. [b] Mann-Whitney test. N, number; TC, total cholesterol, LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; ProCPU, procarboxypeptidase U (TAFI, proCPB2); CLT, clot lysis time; CLT_{PTCI}, CLT in the presence of potato-tuber carboxypeptidase inhibitor (PTCI); ΔCLT; absolute reduction in CLT after addition of PTCI; sTM, soluble thrombomodulin; hsCRP, high-sensitive C-reactive protein.

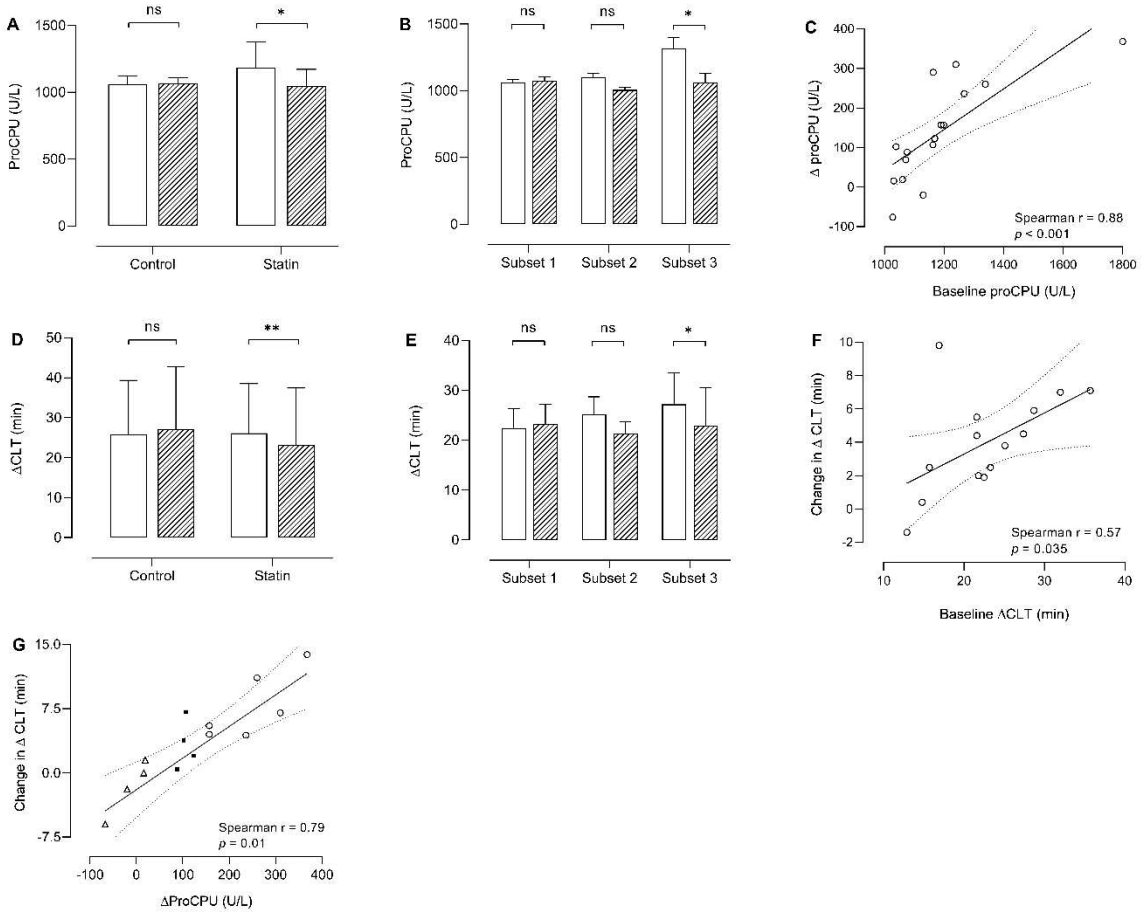

Figure 1 - ProCPU levels (A-C). (A) Bar graph showing plasma procarboxypeptidase U (proCPU) levels in controls (N = 12) and statin-treated patients (N = 16) at baseline (white) and after three months (black stripes). Data presented as mean ± SD. *Wilcoxon Matched-Pairs Signed Rank test; p<0.05. **(B)** Bar graph showing plasma proCPU levels at baseline (white) and after three months (black stripes) in three subsets of statin patients. Subset 1: patients with baseline proCPU levels similar to controls and no proCPU decrease under influence of a statin (N = 4). Subset 2: patients with baseline proCPU levels similar to controls and a 5-10% decrease in proCPU levels after three months of statin use (N = 5). Subset 3: patients in which baseline proCPU levels were 20% higher and in which statin treatment resulted in approximately 20% decrease after three months (N = 7). Data presented as mean ± SD. *Wilcoxon Matched-Pairs Signed Rank test; p<0.05. **(C)** Correlation between baseline proCPU levels and the decrease in proCPU (ΔproCPU) in statin-treated individuals (N = 16). Spearman correlation coefficient r was determined and the best-fit line (solid line) with 95% confidence bands was plotted

(dashed lines). **ΔCLT (actual contribution of the CPU system to the total clot lysis time) (D-F). (D)** Bar graph showing ΔCLT in controls (N = 11) and statin-treated patients (N = 16) at baseline (white) and after three months (black stripes). Data presented as mean ± SD. **Wilcoxon Matched-Pairs Signed Rank test; $p<0.01$. **(E)** Bar graph showing ΔCLT at baseline (white) and after three months (black stripes) in three subsets of statin patients. Subset definitions see panel (B). Data presented as mean ± SD. *Wilcoxon Matched-Pairs Signed Rank test; $p<0.05$. **(F)** Correlation between baseline ΔCLT and the change in ΔCLT in statin-treated individuals (N = 16). Spearman correlation coefficient r was determined and the best-fit line (solid line) with 95% confidence bands was plotted (dashed lines). **Correlation between the change in proCPU levels (ΔproCPU) and the change in ΔCLT in statin-treated individuals (G)**. Spearman correlation coefficient r was determined and the best-fit line (solid line) with 95% confidence bands was plotted (dashed lines). Each symbol represents a different subset: Open triangle – subset 1 (N = 4); Black square – subset 2 (N = 4); Open dot – subset 3 (N = 6).

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