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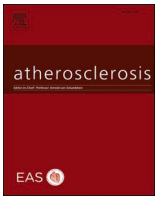

Plasma levels of PRO-C3, a type III collagen synthesis marker, are associated with arterial stiffness and increased risk of cardiovascular death

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ABSTRACT

Background and aims: The N-terminal propeptide of type III collagen (PRO-C3) assay measures a pro-peptide released during type III collagen synthesis, an important feature of arterial stiffening and atherogenesis. There is a clinical need for improved non-invasive, cheap and easily accessible methods for evaluating individuals at risk of cardiovascular disease (CVD). In this study, we investigate the potential of using circulating levels of PRO-C3 to mark the degree of vascular stenosis and risk of cardiovascular events.

Methods: Baseline plasma levels of PRO-C3 were measured by ELISA in subjects belonging to the SUrrogate markers for Micro- and Macro-vascular hard endpoints for Innovative diabetes Tools (SUMMIT) cohort (N = 1354). Associations between PRO-C3 levels with vascular characteristics, namely stiffness and stenosis, and risk of future cardiovascular events were explored. Subjects were followed up after a median of 35 months (interquartile range 34–36 months), with recorded outcomes cardiovascular death and all-cause mortality.

Results: We found a correlation between PRO-C3 levels and pulse wave velocity (*rho* 0.13, $p = 0.000009$), a measurement of arterial stiffness. Higher PRO-C3 levels were also associated with elevated blood pressure (*rho* 0.07, $p = 0.014$), as well as risk of cardiovascular mortality over a three-year follow-up period (OR 1.56, confidence interval 1.008–2.43, $p = 0.046$).

Conclusions: Elevated circulating PRO-C3 levels are associated with arterial stiffness and future cardiovascular death, in the SUMMIT cohort, suggesting a potential value of PRO-C3 as a novel marker for declining vascular health.

1. Introduction

Identification of novel circulatory markers of vascular health would greatly benefit diagnosis, risk-stratification and monitoring of cardiovascular disease (CVD) and allow for simple, fast and cheap evaluation of individuals at risk. One hallmark of arterial stiffening, an early manifestation and marker of declining vascular health, is collagen deposition [1,2], which results in the release of extracellular matrix (ECM) protein fragments into circulation.

Type III collagen expression is lower, and accompanied by a reduced type III/type I collagen ratio and increased elastin/type III collagen ratio, in the aortic wall of patients with acute myocardial infarction compared to healthy control subjects and patients with stable angina [3]. Furthermore, association between turnover of type III, but not type

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I, collagen and arterial stiffening has been described in elderly women with severely elevated systolic blood pressure [4], while both quantitative and qualitative type III collagen defects resulted in reduced aortic stiffening in a mouse model [5]. Type III collagen turnover may thus have potential as a vascular disease marker.

The PRO-C3 assay quantifies the circulating N-terminal pro-peptide of type III collagen, which is released during collagen maturation and is a biomarker of fibrogenesis [6–8]. By targeting the N-protease cleavage site of the N-terminal propeptide, the PRO-C3 assay is specific to type III collagen synthesis [9], as it reflects the active process of release of pro-peptide during type III collagen formation. In this study, we have investigated the association between circulating levels of PRO-C3 and CVD risk factors and events to assess whether PRO-C3 may merit further consideration as a candidate vascular disease marker.

2. Materials and methods

Methods are described in detail in the Supplementary materials.

2.1. Study cohort: the SUrrogate markers for micro- and macro-vascular hard endpoints for innovative diabetes tools (SUMMIT)

The study was carried out in accordance with the principles of the 1975 Declaration of Helsinki and approved by the Swedish Ethical Review Authority. Written informed consent was obtained by all subjects. Sharing of datasets containing de-identified participant data included in this study are subject to limitations specific to the ethical permit and general data protection regulation (GDPR, (EU) 2016/679).

Fifteen hundred subjects were recruited between December 2010 and April 2013 from university hospitals in Malmö (Sweden), Pisa (Italy), Dundee and Exeter (UK), as previously described [10]. Following analysis, 146 subjects were excluded from the complete cohort (n = 1500) due to insufficient absorbance readout quality of plasma PRO-C3 quantification. Patient clinical characteristics are summarized in Supplementary Table 1. Classification of a clinical history of CVD at baseline has been described previously [10].

2.2. Follow-up of subjects

Subjects were followed up after three years at all four sites. Information was gathered of fatal and non-fatal cardiovascular events (defined similarly as at study inclusion) as well as of all-cause mortality. For subsequent regression analysis, PRO-C3 levels were normalized by logarithmic transformation after which z-scores were computed.

2.3. Plasma PRO-C3 analysis

The marker reflecting synthesis of type III collagen (PRO-C3) was measured in EDTA plasma samples by competitive ELISA (CAT No. 1000) developed at Nordic Bioscience (Herlev, Denmark), as described previously [6].

2.4. Arterial stiffness evaluation and ultrasound imaging of the carotid arteries

Arterial stiffness was evaluated through calculation of pulse wave velocity using a Sphygmocor device (Atcor Medical, Australia) as described previously [10,11].

2.5. Statistics

The Mann-Whitney *U* test was used for comparisons of groups, and Spearman's rank correlation and the Chi-square test were used to compare continuous and categorical variables, respectively. Associations with cardiovascular death during the follow up period was performed using a logistic regression model. Regression analyses were corrected for confounders using the following model: age, sex, baseline presence of CVD, diabetes and hypertension. IBM SPSS version 27 and GraphPad Prism Version 9.2.0 (GraphPad Software Inc, CA, USA) were used for statistical analysis.

Table 1
Associations between circulating PRO-C3 levels (at study inclusion) and clinical characteristics.

Clinical characteristics	All n = 1354			
Continuous	Absolute values	rho	*p*	*p*, adjusted[a]
Age (years)	69 (62-74	.078	0.012	0.058
Body mass index	28.5 (25.6–32.2)	0.11	0.000043	0.00034
Total cholesterol (mmol/L)	4.3 (3.6–50.0)	−0.027	0.33	0.70
Triglycerides (mmol/L)	1.3 (10.0–1.9)	0.065	0.017	0.067
LDL[b] (mmol/L)	2.3 (1.8–30.0)	−0.007	0.80	0.92
HDL[c] (mmol/L)	1.3 (10.0–1.5)	−0.10	0.00024	0.0019
HbA1c (mmol/mol)	48 (40–58.5)	0.010	0.71	0.92
eGFR[d] (μmol/L)	80.8 (68.5–930.0)	−0.10	0.00016	0.0011

Categorical			***p***	***p*, adjusted**
Sex (female)	34	–	0.689	0.69
Smoking[e]	11/52/37	–	0.154	0.379
Diabetes	67	–	0.147	0.27
Cardiovascular disease	48	–	0.006	0.020
Hypertension	67	–	0.000063	0.00031

Statistical association was analysed using Spearman's Rho (continuous variables) and the Mann-Whitney test (categorical variables). Continuous measurements are shown as median with interquartile range (IQR; due to non-Gaussian distribution) and categorical variables as percentages.

[a] Adjusted for multiple comparisons using the Holm-Šídák test.
[b] Low-density lipoprotein.
[c] High-density lipoprotein.
[d] Estimated glomerular filtration rate.
[e] Current smoker/ex-smoker/never smoked.

3. Results

3.1. Patient demographics

To investigate associations between type III collagen formation and CVD, we measured the levels of PRO-C3 in plasma taken at study baseline from 1354 subjects belonging to the SUMMIT cohort. The majority of the subjects were male (66%) with a median age of 69 years. At baseline, 67% of the included subjects had a diagnosis of diabetes type 2, 48% of CVD, and 67% were hypertensive. Sixty-six % were treated with statins (Supplementary Tables 1–2).

PRO-C3 levels did not increase with age, and nor were they dependent on sex, but a positive correlation was found with body mass index (rho = 0.11, $p = 0.00034$), while a negative correlation was found with high-density lipoprotein (HDL; rho = −0.10, $p = 0.0019$) and the estimated glomerular filtration rate (eGFR; rho = −0.10, $p = 0.0011$, Table 1). PRO-C3 levels were not associated with type 2 diabetes.

3.2. Circulating PRO-C3 is associated with cardiovascular disease

PRO-C3 levels at study baseline were associated with CVD ($p = 0.020$), as well as unstable angina ($p = 0.020$; Table 1). Though PRO-C3 levels were associated with the number of plaques in both the right and and left common carotid arteries (*rho* = 0.103, $p = 0.000242$; and *rho* = 0.091, $p = 0.001$, respectively), they were not associated with intima-media thickness (IMT) on either side (Supplementary Table 3).

3.3. Circulating PRO-C3 is associated with arterial stiffness

Plasma levels of PRO-C3 were significantly correlated with arterial stiffness as assessed by pulse wave velocity ($p = 0.000009$; Table 2). Consistent with this finding, plasma levels of PRO-C3 correlated with both diastolic and systolic blood pressure (rho = 0.068, $p = 0.014$ and rho = 0.075, $p = 0.014$, respectively). Plasma levels of PRO-C3 were also associated with a diagnosis of hypertension ($p = 0.00031$), even after adjustment for baseline CVD (OR 1.21, CI 1.07–1.30, $p = 0.002$). Furthermore, PRO-C3 plasma levels correlated with augmentation index normalized to heart rate (rho = 0.11, $p = 0.00043$; Table 2).

3.4. Circulating PRO-C3 is associated with future cardiovascular death

Finally, we explored the potential of plasma levels of PRO-C3 to predict future cardiovascular events. We found plasma PRO-C3 levels to be associated with the occurrence of cardiovascular death during a follow-up period of 3 years after adjusting for age, sex, baseline presence of CVD, diabetes, hypertension and smoking (OR 1.56, 95% CI 1.008–2.43, $p = 0.046$; Table 3). PRO-C3 levels were not associated with death of any cause analysed during the same follow-up period and using the same adjustment models (OR 1.24; 95% CI 0.91–1.67, $p = 0.17$; Table 4).

Table 3
Logistic regression analysis of association between the circulating levels of PRO-C3 in the SUMMIT cohort and fatal cardiovascular events (adjusted for confounding factors) over a 3-year follow-up period.

All patients	Cardiovascular death N = 1318 (13 events)		
Adjustment models (z-score)	Odds ratio	Confidence interval	*p*
Unadjusted	1.56	1.052–2.31	0.027
Model 1a: Age, sex, baseline CVD and diabetes	1.59	1.029–2.45	0.037
Model 1b: Model 1a + baseline hypertension + smoking[a]	1.56	1.008–2.43	0.046

[a] Current smoker/ex-smoker/never smoked.

Table 4
Logistic regression analysis of association between the circulating levels of PRO-C3 in the SUMMIT cohort and all-cause mortality (adjusted for confounding factors) over a 3-year follow-up period.

All patients	All-cause mortality N = 1318 (43 events)		
Adjustment models (z-score)	Odds Ratio	Confidence interval	*p*
Unadjusted	1.29	0.99–1.67	0.057
Model 1a: Age, sex, baseline CVD and diabetes	1.27	0.95–1.70	0.10
Model 1b: Model 1a + baseline hypertension + smoking[a]	1.24	0.91–1.67	0.17

[a] Current smoker/ex-smoker/never smoked.

4. Discussion

There is a clinical need to identify individuals at risk of CVD with simple, easily available and cheap methods. With this study, we explored whether the type III collagen synthesis marker PRO-C3 is associated to CVD risk factors or can predict future CVD events. We found an association between elevated plasma PRO-C3 levels in the SUMMIT cohort and arterial stiffness (Fig. 1). Moreover, circulating PRO-C3 levels are associated with the risk of future cardiovascular death.

Type III collagen is an integral component of the healthy artery wall. In the aorta, type I collagen comprises two-thirds of the total collagen content, being especially abundant in the adventitia and diseased intima, while type III collagen makes up the major share of the remaining third, particularly in the media [12] and in regions rich in calcium deposits [13]. Type III collagen is also reportedly increased in the infarct

Table 2
Association between circulating PRO-C3 levels (at study inclusion) and arterial stiffness parameters.

Arterial stiffness	All n = 1101			
	Absolute values	rho	*p*	*p* [a]
Pulse-wave velocity (Δdistance/Δtime)	10.4 (8.8–14.5)	0.13	0.000009	0.000045
Augmentation index (normalized to heart rate)	12.4 (2.8–24.8)	0.11	0.000144	0.00043
Diastolic blood pressure	76 (70–83)	0.07	0.013	0.014
Systolic blood pressure	134 (123–147)	0.07	0.0070	0.014

Continuous measurements are shown as median with interquartile range (IQR; due to non-Gaussian distribution). Statistical association was analysed using Spearman's Rho.

[a] Adjusted for multiple comparisons using the Holm-Šídák test.

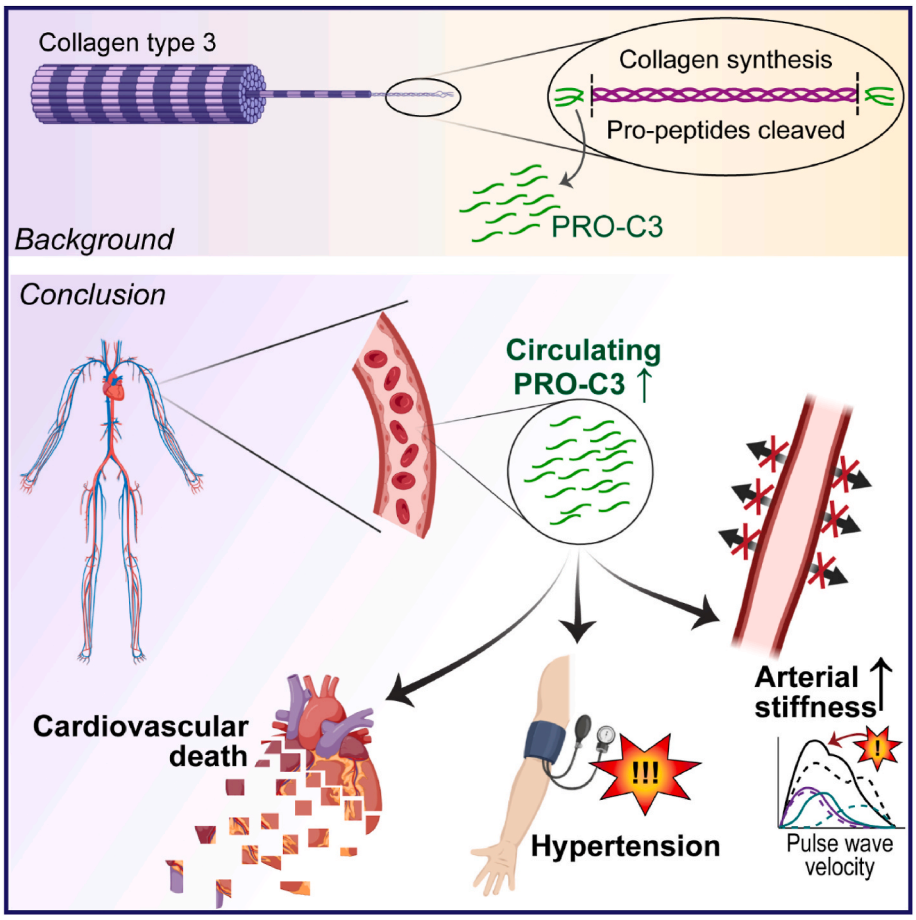

Fig. 1. Graphical abstract.
Elevated levels of circulating PRO-C3, a by-product of type III collagen synthesis, are associated with arterial stiffness, hypertension and future cardiovascular death in the SUMMIT cohort, suggesting a potential value of PRO-C3 as a novel marker for vascular disease. Created in part with BioRender.com.

zone post-myocardial infarction [14]. Genetic variants of the type III procollagen gene, COL3A1, cause vascular Ehlers-Danlos syndrome, where fragile vessels (mainly manifested by low total collagen content and irregular elastic fibrils) increase the risk of aneurysms and arterial dissections [15].

In addition to being an essential element of the vasculature, type III collagen is essential in regulating type I collagen fibrillogenesis [16], and the type III collagen synthesis marker PRO-C3 has been previously linked to fibrotic disease. Increased circulating levels have been reported in patients with chronic hepatitis C [9,17] and idiopathic pulmonary fibrosis [18], with further association to disease progress in both cases, as well as in subjects with chronic obstructive pulmonary disease [19]. Associations were also found between PRO-C3 levels and severity of steatohepatitis and fibrosis stage in adults with non-alcoholic fatty liver disease [20,21]. Moreover, elevated PRO-C3 serum levels were prognostic of poor survival in subjects with metastatic colorectal cancer [22] and pancreatic cancer [23].

We found that circulating PRO-C3 is associated with pulse wave velocity, a measurement used to assess arterial stiffness. Arterial stiffening represents a degenerative process, featuring collagen deposition and cross-linking, as well as elastin fragmentation. Unlike atherosclerosis, which develops in the intimal vessel layer, arterial stiffening mainly involves the media [24]. Arterial stiffness impacts cardiovascular health in several ways: it causes hypertension and promotes left ventricular remodelling and dysfunction. Arterial stiffness further leads to increased pressure in the microcirculation, particularly damaging organs relying on high microcirculatory flow, such as the brain and kidneys. Though arterial stiffness increases with normal aging, it is also affected by e.g., dyslipidaemia, smoking, obesity and diabetes mellitus [25].

In line with our finding that increasing PRO-C3 levels in plasma are associated with an increased risk of cardiovascular death (within three years), aortic stiffness has been identified as an independent predictor of cardiovascular mortality [26]. The potential use of PRO-C3 as a prognostic proxy for aortic stiffness for this purpose may be of interest, as – where evaluation of aortic stiffness requires specialized equipment to measure pulse wave velocity – PRO-C3 levels may be analysed in a pre-existing blood test. So far there is no validated, nor clinically used, biomarker for arterial stiffness and other proposed candidate markers are described mainly in smaller cohorts (<200 subjects [27–33]) than our current study. Considering our comparably sizeable cohort, PRO-C3 – with robust validation and clinical testing – may be of use in evaluating arterial stiffness in situations where pulse wave velocity measurements are unavailable, a notion possibly also of value in research settings, e.g., retro- and prospective studies using cohorts where blood has been collected.

Interestingly, though associations have been reported between arterial stiffness and atherosclerotic burden [24], as confirmed also in the presently investigated SUMMIT cohort (Supplementary Table 4), circulating PRO-C3 was associated with cardiovascular mortality only, not the degree of arterial injury. This suggests that PRO-C3 may specifically provide information on the mechanical state of arteries, independent of the presence of atherosclerosis. Importantly, we cannot, through the current study, infer whether PRO-C3 levels measured in plasma represent purely a reflection of the stiffness status of arteries, or if PRO-C3 also has a causative role in the process of arterial stiffening.

Sixty-seven percent of the present cohort were diagnosed with hypertension at inclusion, with the vast majority also undergoing antihypertensive treatment. Claridge et al. reported a higher mean collagen turnover among subjects undergoing specifically angiotensin-converting enzyme inhibitor (ACE-I) therapy, and levels of the collagen turnover marker type III procollagen peptide, or PIIINP, were then associated with ACE-I therapy [34]. In our cohort, we found plasma levels of PRO-C3 to be associated with use of blood pressure-lowering pharmacotherapy, in particular beta blockers (data not shown; both $p > 0.001$). However, our reported association between PRO-C3 and cardiovascular mortality did remain significant among hypertensive subjects also when adjusting for such treatment (data not shown).

Type III collagen has been found to correlate with the total microvessel content in human aortic atherosclerotic plaques [35]. Taking into account the critical role of type III collagen in vessel fibrillogenesis, a process also driving arterial stiffening, it is not unlikely that, when arterial stiffening reaches a pathological degree, a significant portion of the overall type III collagen synthesis is involved in this process. Moreover, hypoxic conditions can result in the induction of angiogenesis, a process that would further involve the synthesis of type III collagen and subsequent release of PRO-C3 to the circulation [36]. Angiogenesis itself has also been directly linked to arterial stiffness through the observation that gene expression of angiopoietins 1 and 2 in monocytes from untreated hypertensive subjects correlated with pulse wave velocity/arterial stiffness [37]. Notably, elevated serum PRO-C3 levels has been described in subjects with pulmonary arterial hypertension [38].

Possible limitations to this study are that the survival analyses cannot account for the exact number at risk due to lack of a registered time variable and that pulse wave velocity measurements are unavailable for a subset of the full cohort (131 of 1354 subjects, i.e., 9.6%). The reasons are nearly impossible to pinpoint but may pose a source of bias if related to the health status of the subjects and it is not possible to know the direction of a potential bias. Furthermore, sex-ratios are skewed towards men (66% of the cohort) and the analyses are not stratified by sex, as power is considerably lower in the female group. However, PRO-C3 levels did not differ between men and women, making significant bias less likely. Of note, based on inclusion ages, women in the cohort are post-menopausal, which limits generalisation of our results regarding younger women (as associations between PRO-C3 levels and sex hormones are currently unknown). Additionally, generalisability of our results will require studies including broader ethnicities and extra European populations.

One should also keep in mind that only associations were studied, and no mechanistic nor causal inference can thus be drawn. Furthermore, as PRO-C3 levels was measured in the circulation, the location/s/ of active type III collagen synthesis cannot be inferred by our study. Though the strong correlation with pulse wave velocity does suggest involvement in processes related to arterial stiffness, active type III collagen synthesis may also occur up- or downstream of processes affecting arterial stiffening or even outside the vasculature, possibly resulting in a potential bias in overestimating the effect of elevated PRO-C3 levels.

Finally, it is important to note that, though we found associations between PRO-C3 levels and prevalent CVD, we cannot exclude that the main association is with arterial stiffness, while associations with CVD may be secondary (and possibly not independent) to arterial stiffness. In addition, it is not known whether circulating PRO-C3 levels merely reflect arterial stiffness status, or if the fragment itself also possesses bioactive properties with a causative role in the stiffening of arteries. A bioactive fragment may also indirectly affect the process of arterial stiffening through signalling feedback loops, risking introduction of a potential overestimation bias in interpreting the relationship between PRO-C3 and arterial stiffening.

Markers of CVD – including by-products of protein turnover reflecting pathological processes, such as type III collagen – that may be evaluated through a blood test represent a new approach to aid in diagnostics, risk-stratification, monitoring and/or prognostics than measurements requiring specific equipment or invasive strategies. With this study we suggest PRO-C3 as a possible non-invasive, novel marker, with the potential to identify arterial stiffness and CVD prognosis in this cohort, warranting further studies.

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CRediT authorship contribution statement

Federica Genovese: contributed to the analysis and/or interpretation of data, designed experiments and conceptualized the study, and all co-authors edited and reviewed the manuscript. **Isabel Gonçalves:** onducted experiments/acquired data, contributed to the analysis and/or interpretation of data, designed experiments and conceptualized the study. **Signe Holm Nielsen:** conducted experiments/acquired data, designed experiments and conceptualized the study. **Morten A. Karsdal:** conducted experiments/acquired data, designed experiments and conceptualized the study. **Andreas Edsfeldt:** contributed to the analysis and/or interpretation of data. **Jan Nilsson:** onducted experiments/acquired data, contributed to the analysis and/or interpretation of data. **Angela C. Shore:** onducted experiments/acquired data, designed experiments and conceptualized the study. **Andrea Natali:** onducted experiments/acquired data, designed experiments and conceptualized the study. **Faisel Khan:** onducted experiments/acquired data, designed experiments and conceptualized the study. **Annelie Shami:** onducted experiments/acquired data, contributed to the analysis and/or interpretation of data, designed experiments and conceptualized the study, wrote the original draft.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

FG, SHN and MAK are full-time employees and shareholders at Nordic Bioscience, the company that produces and holds patents for the PRO-C3 assay. All other authors have no conflict of interest to report.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2023.117420.

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