Reduced vitamin K status as a potentially modifiable risk factor of severe COVID-19

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Summary: Indirectly quantified extrahepatic vitamin K status is severely reduced in COVID-19 patients. Data suggest pneumonia-induced vitamin K depletion leading to accelerated elastic fiber damage and thrombosis risk due to impaired vitamin K-dependent activation of MGP and endothelial protein S, respectively.

Abstract

Background Respiratory failure and thromboembolism are frequent in SARS-CoV-2-infected patients. Vitamin K activates both hepatic coagulation factors and extrahepatic endothelial anticoagulant protein S, required for thrombosis prevention. In times of vitamin K insufficiency, hepatic procoagulant factors are preferentially activated over extrahepatic proteins. Vitamin K also activates matrix Gla protein (MGP), which protects against pulmonary and vascular elastic fiber damage. We hypothesized that vitamin K may be implicated in coronavirus disease 2019 (COVID-19), linking pulmonary and thromboembolic disease.

Methods 135 hospitalized COVID-19 patients were compared with 184 historical controls. Poor outcome was defined as invasive ventilation and/or death. Inactive vitamin K-dependent MGP (dp-ucMGP) and prothrombin (PIVKA-II) were measured, inversely related to extrahepatic and hepatic vitamin K status, respectively. Desmosine was measured to quantify the rate of elastic fiber degradation. Arterial calcification severity was assessed by computed tomography.

Results Dp-ucMGP was elevated in COVID-19 patients compared to controls ($p<0.001$), with even higher dp-ucMGP in patients with poor outcomes ($p<0.001$). PIVKA-II was normal in 82.1% of patients. Dp-ucMGP was correlated with desmosine ($p<0.001$), and coronary artery ($p=0.002$) and thoracic aortic ($p<0.001$) calcification scores.

Conclusions Dp-ucMGP was severely increased in COVID-19 patients, indicating extrahepatic vitamin K insufficiency, which was related to poor outcome while hepatic procoagulant factor II remained unaffected. These data suggest a mechanism of pneumonia-induced extrahepatic vitamin K depletion leading to accelerated elastic fiber damage and thrombosis in severe COVID-19 due to impaired activation of MGP and endothelial protein S, respectively. A clinical trial could assess whether vitamin K administration improves COVID-19 outcomes.

Keywords: COVID-19; elastic fibers; factor II; matrix Gla protein; protein S; vitamin K

Introduction

Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome (SARS) coronavirus (CoV)-2 [1]. The majority of individuals who contract SARS-CoV-2 have mild symptoms, however, a significant proportion develops respiratory failure due to pneumonia [1]. COVID-19 may also have extrapulmonary manifestations including coagulopathy and venous thromboembolism, which are associated with decreased survival [2]. The mechanisms that activate coagulation in COVID-19 remain incompletely understood.

Coagulation is an intricate balance between clot promoting and dissolving processes in which vitamin K plays a well-known role. Procoagulant factor II (FII; *i.e.* prothrombin) requires vitamin K-dependent carboxylation to fulfil its primary function. Vitamin K is also cofactor of anticoagulant protein S. In contrast to FII, a significant proportion of protein S is extrahepatically synthesized in endothelial cells, which plays a local suppressive role against thrombosis [3]. Vitamin K deficiency results in more severely compromised carboxylation of extrahepatic than of hepatic vitamin K-dependent proteins (figure 1) [4]. This can paradoxically lead to enhanced thrombogenicity in a state of low vitamin K [5].

Matrix Gla protein (MGP) is also vitamin K-dependent but not involved in coagulation [6]. MGP is well-known as a calcification inhibitor in arterial walls [7], but MGP's role in the pulmonary compartment seems to be comparable [8, 9]. Elastic fibers are essential matrix components in lungs and have high calcium affinity [10]. Degradation and mineralization of elastic fibers are interrelated processes [11, 12]. Matrix metalloproteinases (MMPs) synthesis increases parallel with elastic fiber calcification [13], and partially degraded elastic fibers become prone to mineralization [10]. Recent data show that a subset of MMP-producing macrophages is increased in severe SARS-CoV-2 pneumonia [14]. COVID-19 may theoretically be linked to both vitamin K deficiency and elastic fiber metabolism through a series of sequential pathologic steps (figure 2).

Individuals with severe SARS-CoV-2 infections often have comorbidities that are associated with reduced vitamin K status, such as hypertension, diabetes, and cardiovascular diseases [1, 7]. The body uses vitamin K very efficiently, and storage capacity is low [15]. There are reasons to suspect that there is increased utilization of vitamin K for carboxylation of pulmonary MGP and coagulant factors during COVID-19 [16]. Vitamin K depletion may have devastating consequences in lungs [17].

The aim of this study was to evaluate whether a reduced vitamin K status plays a role in the pathogenesis of COVID-19 through interacting with both elastic fiber metabolism and the coagulation cascade, thereby linking pulmonary and coagulopathic disease manifestations.

Methods

Subjects

135 subjects hospitalized for COVID-19 in the Canisius-Wilhelmina Hospital between March 12th and April 15th 2020 were included. SARS-CoV-2 infection was confirmed by Real Time polymerase chain-reaction testing. Patient data was extracted from hospital records, and vitamin K antagonist (VKA) usage was determined from pharmacy and anticoagulant clinic records. The study was approved by the United Medical Research Ethics Committees of the Canisius-Wilhelmina Hospital (CWZ-nr. 027-2020; date of approval 12th March 2020), which waived the need for written informed consent. Patients could opt-out after they were informed about the study.

186 control subjects from a previously published COPD study were included in addition (www.controlled-trials.com, identifier ISRCTN86049077) [18]. Two control subjects where the use of VKA was unknown were excluded from the analysis.

Patients were followed-up until: 1) discharge from the hospital, 2) intubation and mechanical ventilation, or 3) death. Outcome of COVID-19 patients was categorized as “good” if they were discharged from the hospital without the need for invasive ventilation, and “poor” if they either

required intubation and mechanical ventilation or died. During admission blood was sampled three times per week and EDTA plasma and serum were frozen at -80°C for retrospective analysis.

Dp-ucMGP

Direct quantification of blood vitamin K is not appropriate to assess vitamin K status due to differences in bioavailability and half-life time between the two naturally occurring forms (vitamin K1 and K2). Additionally, the intake of vitamin K2 is too low to measure accurately. Measuring inactive levels of vitamin K-dependent protein in the circulation is a valuable method to quantify the combined deficit of vitamin K1 and K2. Desphospho-uncarboxylated (dp-uc)MGP (inactive MGP) is an appropriate indirect marker of extrahepatic vitamin K status [19, 20]. Subjects with high dp-ucMGP levels have low extrahepatic vitamin K status and *vice versa*.

Circulating dp-ucMGP levels were determined in EDTA plasma using the commercially available IVD chemiluminescent InaKif MGP assay on the IDS-iSYS system (IDS, Boldon, UK) as previously described [21]. The within-run and total precision of this assay were 0.8–6.2% and 3.0–8.2%, respectively. The assay measuring range is between 200–12,000pmol/L and was found to be linear up to 11,651pmol/L. Dp-ucMGP values <300pmol/L are in the normal healthy range and values >500pmol/L reflect vitamin K deficiency [22].

PIVKA-II

Protein induced by vitamin K absence (PIVKA)-II was used to assess hepatic vitamin K status. Subjects with high PIVKA-II levels have low hepatic vitamin K status and *vice versa*. Circulating PIVKA-II levels were measured in serum using a conformation-specific monoclonal antibody in an ELISA-based assay as previously described [23]. The detection limit, as well as upper limit of normal, was 0.15AU/mL [23].

Desmosine

Plasma (p)desmosine and isodesmosine (DES) levels were used as a marker for the rate of elastic fiber degradation [24]. DES are formed during the cross-linking of tropo-elastin polymers and are released in the bloodstream after degradation of elastic fibers [24]. pDES directly reflects the rate of systemic elastic fiber degradation.

DES fractions were measured using liquid chromatography-tandem mass spectrometry as previously described [18, 24]. Coefficient of variations of intra- and inter-assay imprecision were <8.2%, lower limit of quantification of 140ng/L, and assay linearity up to 210,000ng/L.

CT assessment

Thin slice CT scans were acquired by using a Philips Ingenuity multi-detector row scanner (Philips Healthcare). CT images of 1-mm thickness were reconstructed by using iterative model-based reconstruction in the axial plane.

Quantitative measurements of the volume of ground glass and consolidation were undertaken using the Intellispace Portal (COPD package, Intellispace version 10, Philips Healthcare). In the software, first the lungs were segmented from the chest wall and major vessels and main bronchi. Manual adjustments were implemented where required by a board-certified chest radiologist. Subsequently, the lung voxels were counted to derive a total lung volume in milliliters. Diseased lung tissue was defined as those voxels with an attenuation of Hounsfield Units (HU) > -700 as previously defined. The abnormal voxels were expressed as a percentage diseased lung of the total volume. HU value at the 85th percentile was used [25, 26].

Coronary and thoracic aortic calcification (CAC and TAC, respectively) were also quantified in the Intellispace Portal (Heartbeat CS package). Calcifications were defined as areas with a HU of 130 and higher. The calcifications were visually localized up to the arterial wall by a board-certified chest

radiologist and semi-automatically segmented. The volume was used as a measure of calcification burden.

Statistical analysis

Statistical analyses were performed using SPSS (version 24, IBM, Chicago, IL, USA). Analysis of variance (ANOVA) was used to compare dp-ucMGP, pDES and radiological scores between groups. Analysis of covariance (ANCOVA) was used to perform aforementioned analyses: dp-ucMGP, CAC and TAC adjusted for age, sex, and use of VKA, and pDES adjusted for age.

For each pDES measurement in a COVID-19 patient, virtual age-matched pDES values were calculated using published pDES equations for never and (ex-)smokers [24]. pDES is strongly dialyzed (R. Janssen, unpublished data), and patients receiving dialysis at baseline were excluded from pDES analyses.

Spearman's correlation coefficient was used to test the association of closest time-matched dp-ucMGP with pDES and radiological scores.

For PIVKA-II, patients were categorized as follows: normal <0.15AU/mL, mildly elevated 0.15-0.5AUmL, moderately elevated 0.5-2.0AU/mL and severely elevated >2.0AU/mL.

Dp-ucMGP, pDES, and radiological scores had a log-normal distribution and were therefore natural log-transformed prior to analyses. Since CAC and TAC scores included values equal to zero, these values were transformed using Ln(CAC+1) and Ln(TAC+1), respectively. The mean difference and 95% Confidence Interval (CI) of log-transformed values was back-transformed to the mean fold change. Normally distributed continuous variables are presented as mean ± standard deviation (SD), whereas continuous variables with a natural-log distribution were presented as back-transformed mean and 95% CI. A P-value of <0.05 was used as threshold for statistical significance.

Results

The mean age of COVID-19 patients was 68±12 years, 93 (69%) were male, and 12 (8.9%) used VKA. Of the historical controls, 85 (46%) were male, 3 subjects (1.6%) were taking VKA, and mean age was 61±6.5 years. Patient and control characteristics are shown in Table 1.

Dp-ucMGP

Dp-ucMGP was measured in all available samples. Maximum dp-ucMGP levels were significantly higher in COVID-19 patients (1476pmol/L, 95% CI, 1341 to 1625) compared to healthy controls (471pmol/L, 95% CI, 434 to 511, mean fold change 3.14, 95% CI, 2.76 to 3.56, p<0.001, figure 3A), which remained significant after adjustment for age, sex, and use of VKAs (p<0.001). Dp-ucMGP levels were significantly higher in COVID-19 patients with poor outcome (1998pmol/L, 95% CI, 1737 to 2296) compared to those with good outcome (1157pmol/L, 95% CI, 1022 to 1312, mean fold change 1.73, 95% CI, 1.43 to 2.08, p<0.001; figure 3A), and significance was maintained after adjustments (p<0.001).

PIVKA-II

PIVKA-II was measured in the first available sample after admission. Levels were normal in 82.1%, mildly elevated in 13.0%, moderately in 4.1% and severely in 0.8% of COVID-19 patients not using VKA (figure 3B). PIVKA-II distribution was comparable between patients with good (78.6%, 15.7%, 4.3% and 1.4%, respectively) and poor outcomes (86.8%, 9.4%, 3.8% and 0%, respectively). PIVKA-II levels were severely elevated in 100% of COVID-19 patients using VKA.

Desmosine

Sufficient plasma for pDES measurements was available for 127 patients and measured in the first available sample after admission. Three dialysis-dependent patients were excluded from the analysis. pDES levels were significantly higher in COVID-19 patients (380ng/L, 95% CI, 355 to 405) compared to age-dependent reference values of never-smokers (243ng/L, 95% CI, 228 to 260; mean fold change 1.56, 95% CI, 1.42 to 1.71, p<0.001) and former or current smokers (278ng/L, 95% CI, 260 to 296, mean fold change 1.37, 95% CI 1.25 to 1.50, p<0.001; figure 4A) [24]. pDES levels, corrected for age, were significantly higher in COVID-19 patients with poor (430ng/L, 95% CI 384 to 481) compared to good outcomes (342ng/L, 95% CI 310 to 379; mean fold change 1.25, 95% CI, 1.07 to 1.47, p=0.004). Dp-ucMGP levels significantly correlated with pDES (n=124, r=0.47, p<0.001; figure 4B).

CT assessment

CT scans were available for 109 patients, and CAC and TAC scores were successfully determined for 107 of these patients. TAC and CAC scores were significantly higher in COVID-19 patients with poor outcome compared to those with good outcome, however, both lost significance after adjustments (supplementary data). The association between pulmonary involvement on CT and time-matched dp-ucMGP levels was not significant (n=109; r=0.18; p=0.06). Dp-ucMGP was significantly associated with TAC scores (n=107; r=0.36; p<0.001) and CAC scores (n=107; r=0.30; p=0.002).

Discussion

Dp-ucMGP, which indirectly indicates extrahepatic vitamin K insufficiency, was severely elevated in hospitalized COVID-19 patients. Impaired MGP activation associated with poor outcome and accelerated elastic fiber degradation. In contrast, procoagulant FII activity remained preserved.

Dp-ucMGP as measure of extrahepatic vitamin K status is a relevant parameter given its close association with mortality [22]. Low dietary vitamin K intake and VKA use are evident causes of elevated dp-ucMGP [15, 22]. Pathological processes leading to upregulation of vitamin K-dependent protein production and resulting in accelerated utilization of vitamin K for carboxylation may be another important reason for severe extrahepatic vitamin K insufficiency in COVID-19. Vitamin K supplementation reduced dp-ucMGP in various cohorts [22, 27], with favorable effects on clinically relevant outcome measurements [27]. It is reasonable to assume that vitamin K administration reduces dp-ucMGP in COVID-19. Whether improving dp-ucMGP results in better outcome of COVID-19 remains to be evaluated.

Intriguingly, many comorbid conditions related to poor COVID-19 clinical outcomes are associated with compromised vitamin K status [1, 7, 18]. The same holds true for ageing [1]. Elastic fiber calcification and degradation are closely related processes [11, 12]. There seems to be an association between vascular mineralization and lung pathologies [28-30]. We demonstrated accelerated elastic fiber degradation and arterial calcification correlating with dp-ucMGP, suggesting interrelationships between vitamin K shortage, insufficient MGP carboxylation and elastic fiber pathologies in COVID-19.

Although significance was lost after adjustment for age, sex, and VKA use, we found enhanced TAC and CAC in patients with poor prognosis. Hypertension, diabetes, cardiovascular disease, and older age are associated with remodeling of elastic tissues [7]. These damaged and calcified elastic fibers are more prone to further degradation than intact fibers [11]. We speculate that this pre-existing

elastic fiber dysfunction renders them more susceptible to degradation following enhanced proteolytic activity during COVID-19 [14].

We did not find a significant correlation between dp-ucMGP and pneumonia severity. It is possible that the correlation is confounded by the fact that those with pre-existing conditions are predisposed to both higher dp-ucMGP and the development of respiratory failure with less pulmonary involvement. Furthermore, CT severity is a dynamic process that may change rapidly [31]. A clinical trial in which change of both vitamin K status and CT severity are simultaneously assessed before and after vitamin K supplementation would be a more suitable analysis.

Vitamin K1, the main source of vitamin K in The Netherlands, is preferentially transported to the liver, implying that the grade of carboxylation is usually higher for hepatic than extrahepatic vitamin K-dependent proteins (figure 1) [3, 4, 32]. This likely explains why dp-ucMGP was severely elevated, while PIVKA-II was normal in the majority of patients. Similar to MGP, the activation of endothelial protein S is disproportionally impacted in times of vitamin K deficiency. Theoretically, these observations could be compatible with enhanced thrombogenicity in COVID-19 [2], where autopsies revealed bilateral deep venous leg thrombosis in all thromboembolic cases, and thrombosis of the prostatic venous plexus in the majority of men who died [33]. Future research should investigate this, however, there is currently no readily available assay to measure carboxylated (active) versus uncarboxylated (inactive) protein S. Enhanced thrombosis in a state of vitamin K deficiency has previously been described in calciphylaxis [5]. Calciphylaxis is characterized by cutaneous blood vessel occlusion due to calcification, leading to ischemic skin infarction [5]. Increased levels of inactive MGP are found in skin tissues and the circulation of calciphylaxis patients [5]. It may be speculated that, analogous to calciphylaxis, impaired local anticoagulant activity due to vitamin K insufficiency is responsible for microvessel thrombosis in COVID-19 [34].

The major strength of our study is the use of robust biomarkers and quantitative CT assessment. Our findings are limited by the fact that it is impossible to determine which proportion of circulating dp-

ucMGP and DES levels originated from the lungs, as both biomarkers are not tissue specific. Therefore, there is urgent need for experimental data to better link vitamin K insufficiency specifically with COVID-19-related lung pathologies.

As low vitamin K levels are found in comorbidities that are related to poor outcome of COVID-19 [1, 7], we were unable formally to determine whether vitamin K insufficiency truly predisposes patients to the development of severe COVID-19 or whether it is merely an epiphenomenon. However, the latter seems highly unlikely given the extreme elevation of dp-ucMGP levels in COVID-19 patients, which was much more pronounced than in hypertensive, diabetic, cardiovascular and COPD patients without COVID-19 (supplementary table 1). The strong correlation that we found between vitamin K status and the rate of elastic fiber degradation also suggests causality.

We had to make use of a historical control group, due to the implementation of quarantines and social distancing practices to contain SARS-CoV-2. We do not consider this to be a significant problem, however, as dp-ucMGP levels of our historical controls were higher than previously reported in large groups of controls (supplementary table 2). Furthermore, differences in dp-ucMGP levels between COVID-19 patients and controls were of such a magnitude that loss of significance when comparing to a matched control group would be highly unlikely.

In conclusion, dp-ucMGP was strongly elevated in hospitalized COVID-19 patients, which indirectly indicates extrahepatic vitamin K insufficiency. Impaired MGP activation was associated with poor outcomes. COVID-19 patients with premorbid elastic fiber pathologies appeared, in particular, to be at increased risk of complicated disease course. Despite extrahepatic vitamin K deficiency, hepatic prothrombin activation remained preserved. Taken together these data suggest a mechanism of pneumonia-induced extrahepatic vitamin K depletion leading to accelerated elastic fiber degradation and thrombosis formation. An intervention trial is now needed to assess whether vitamin K administration improves outcome in patients with COVID-19 by increasing pulmonary MGP and endothelial protein S activation.

Authors' contributions

RJ developed the theory. ASMD, RJ and LJS designed the study. LJS, PL and CM were responsible for the dp-ucMGP and PIVKA-II; and JMWO and HD for the DES measurements. PAJ performed the CT analyses. HD, EGAK, CV, MPJV and JW analyzed the data, and IP performed the statistical analysis. IP, JW and RJ wrote the first draft of the manuscript. ASMD, LJS, JMWO, PAJ, TMH, RG, LEMK, and EFMW critically revised the manuscript.

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Conflict of interest statements

LJS reports consultancy fee from Immunodiagnostic systems and grants from NattoPharma. JMWO and RJ are owners of Desmosine.com and RJ is owner of Emphysema Solutions BV. RJ discloses application of a patent on vitamin K in COVID-19 for prognostic and therapeutic purposes. ASMD, IP, JW, MPJV, TMH, PAJ, RG, PL, HD, CM, EGAK, LEMK, CV, and EFMW declare no competing interests.

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Tables and figures

Table 1: Baseline characteristics of COVID-19 patient and healthy control cohorts.

	COVID-19			Controls
	Good outcome	Poor outcome	All	
	N (%)	N (%)	N (%)	
Subjects	75	60	135	184
Age (years)	64±13	72±10	68±12	61±6.5
Male (%)	46 (61)	47 (78)	93 (69)	85 (46)
VKA use (%)	5 (6.7)	7 (12)	12 (8.9)	3 (1·6)
Hypertension (%)	28 (37)	21 (35)	49 (36)	41 (22)
Diabetes mellitus (%)	15 (20)	15 (25)	30 (22)	14 (7.6)
Cardiac or cardiovascular disease (%)	17 (23)	21 (35)	38 (28)	12 (6.5)
Asthma/COPD (%)	13 (17)	12 (20)	25 (19)	7 (3.8)
Other respiratory disease (%)	5 (6.7)	10 (17)	15 (11)	3 (1.6)
Immunocompromised (%)	4 (5.3)	2 (3.3)	6 (4.4)	0 (0)
Dialysis dependent (%)*	1 (1.3)	2 (3.3)	3 (2.2)	0 (0)
Active malignancy (%)	6 (8.0)	6 (10)	12 (8.9)	0 (0)

COVID-19: Coronavirus 2019; *VKA*: Vitamin K antagonist; *COPD*: chronic obstructive pulmonary disease

* At admission

FIGURE LEGENDS

Figure 1. Distribution of vitamin K1 in the body

(1) After absorption, vitamin K1 is preferentially transported to the liver via the portal circulation, where it is utilized for carboxylation of hepatic coagulation factors. This implies that during periods of vitamin K insufficiency, (2) the grade of carboxylation is usually higher for hepatic factor II (3) than for endothelial protein S in veins and pulmonary matrix Gla protein (MGP).

Figure 2. Proposed sequential pathologic steps linking SARS-CoV-2 pneumonia to vitamin K insufficiency and accelerated elastic fiber degradation

(1) Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) enters alveolar type II (AT2) cell. (2) The infected AT2 cell responds by upregulating synthesis of proinflammatory cytokines. (3) This leads to an increase in the number and activation of pulmonary macrophages. (4) These infiltrating macrophages produce matrix metalloproteinases (MMPs) (5), which leads to accelerated degradation of elastic fibers (5a) and thereby the release of desmosine from these fibers (5b) leading to elevated desmosine levels in lungs and blood. (6) The increased polarity of partially degraded elastic fibers (7) enhances their affinity for calcium, and consequently, leads to increased elastic fiber calcium content. (7a) MMP synthesis is upregulated in parallel with calcium content, which further accelerates elastic fiber degradation in a self-propagating vicious circle. (8) Matrix Gla protein (MGP) synthesis is upregulated in an attempt to protect elastic fibers from calcification and degradation, (8a) which means that need for vitamin K to activate additional MGP increases. (8b) This increased utilization of vitamin K may induce vitamin K insufficiency, (9) in which case increased production of MGP in a state of vitamin K insufficiency leads to increased desphospho-uncarboxylated (dp-uc)MGP in lungs and blood.

Figure 3: Circulating dp-ucMGP and PIVKA-II in COVID-19 patients. (A) Dp-ucMGP was measured in plasma of COVID-19 patients with a good outcome (discharge without invasive ventilation, n=75, orange) or poor outcome (invasive ventilation and/or death, n=60, red), compared to a cohort of controls. Subjects with high dp-ucMGP have low extrahepatic vitamin K status and *vice versa*. The maximal dp-ucMGP measured during the study is shown, with open circles representing those patients using VKA at admission. **(B)** PIVKA-II was measured in plasma at baseline in those patients not using VKA (n=122). The detection threshold and normal range for healthy controls is shown in gray. A single patient not using VKAs had a severely elevated PIVKA-II outside the detection range and is not shown in the figure.

Figure 4: Correlation between dp-ucMGP and desmosine. (A) Scatterplot showing circulating desmosine levels in those patients over 40 years old (n=121) by age, the black line represents the deduced equation for COVID-19 patients. The green and blue lines represent Huang *et al*'s calculated equations for non-smoking and smoking controls, respectively. **(B)** For all COVID-19 patients who were not dialysis dependent at admission with a good outcome (discharge without invasive ventilation, n=69, orange) or poor outcome (invasive ventilation and/or death, n=58, red) log-transformed baseline dp-ucMGP and desmosine values are shown, with open circles representing VKA users. The black line represents a linear regression analysis.