Novel insights into osteogenesis and matrix remodelling associated with calcific uraemic arteriolopathy

Rafael Kramann1,2*, Vincent M. Brandenburg3*, Leon J. Schurgers4, Markus Ketteler5, Saskia Westphal2, Isabelle Leisten2, Manfred Bovi2, Willi Jahnen-Dechent6, Ruth Knüchel2, Jürgen Floege1 and Rebekka K. Schneider2

1Division of Nephrology and Clinical Immunology, RWTH Aachen University, Aachen, Germany, 2Institute of Pathology, RWTH Aachen University, Aachen, Germany, 3Department of Cardiology, RWTH Aachen University, Aachen, Germany, 4Department of Biochemistry, Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht, The Netherlands, 5Department of Medicine III, Division of Nephrology, Klinikum Coburg, Coburg, Germany and 6Institute for Biomedical Engineering, Biointerface Laboratory, Aachen, Germany

Correspondence and offprint requests to: Rafael Kramann; E-mail: rkramann@gmx.net

*R.K. and V.M.B. are contributed equally to this work.

ABSTRACT

Background. Calcific uraemic arteriolopathy (CUA) or calciphylaxis is a rare, life-threatening disease predominantly occurring in patients with end-stage renal disease. Its pathogenesis has been suggested to include ectopic osteogenesis in soft tissue and the vasculature associated with extracellular matrix (ECM) remodelling.

Methods. To gain further insights into the pathogenesis of CUA, we performed systematic analyses of skin specimens obtained from seven CUA patients including histology, immunohistochemistry, electron microscopy, electron dispersive X-ray analysis (EDX) and quantitative real-time RT-PCR. Skin specimens of (i) seven patients without chronic kidney disease and without CUA and (ii) seven dialysis patients without CUA served as controls.

Results. In the CUA skin lesions, we observed a significant upregulation of bone morphogenic protein 2 (BMP-2), its target gene Runx2 and its indirect antagonist sclerostin. Furthermore, we detected an increased expression of inactive uncarboxylated matrix Gla protein (Glu-MGP). The upregulation of osteogenesis-associated markers was accompanied by an increased expression of osteopontin, fibronectin, laminin and collagen I indicating an extensive remodelling of the subcutaneous ECM. EDX analysis revealed calcium/phosphate accumulations in the subcutis of all CUA patients with a molar ratio of 1.68 ± 0.06 matching that of hydroxyapatite mineral. Widespread media calcification in cutaneous arterioles was associated with destruction of the endothelial layer and partial exfoliation of the endothelial cells (ECs). CD31 immunostaining revealed aggregates of ECs contributing to intraluminal obstruction and consecutive malperfusion resulting in the clinical picture of ulcerative necrosis in all seven patients.

Conclusions. Our data indicate that CUA is an active osteogenic process including the upregulation of BMP-2 signalling, hydroxyapatite deposition and extensive matrix remodelling of the subcutis.

INTRODUCTION

Calcific uraemic arteriolopathy (CUA) or calciphylaxis is a rare, life-threatening disease predominantly affecting patients
with end-stage renal disease (ESRD) or chronic kidney disease (CKD). It is characterized by media calcification of small- and medium-sized cutaneous vessels, soft-tissue calcification and clinically by progressive painful skin indurations with ulcerations [1, 2]. The term calciphylaxis was introduced by Hans Selye in the early 1960s [3] based on a rodent model of systemic and local soft-tissue calcifications. Selye postulated a two-step hypothesis with sensitization factors (i.e. hyperparathyroidism, vitamin D or a diet high in calcium and phosphorus) followed by challenging factors (i.e. local trauma, or the application of iron salt, polymycin, egg albumin or glucocorticoids). Subsequently, a similar syndrome with peripheral ischaemic tissue necrosis and vascular calcification was reported in a woman with acute renal failure and this was also named calciphylaxis [4]. However, the experimental model of Selye was free of vascular calcification and, therefore, the human disease is more appropriately described by the term CUA [5]. The prevalence of CUA is nowadays estimated to range from less than 1–4% among ESRD patients [1, 6, 7].

The pathogenesis of CUA remains incompletely understood. Multiple clinical risk factors such as female gender, diabetes, obesity, warfarin use, hyperphosphatemia, hyperparathyroidism, the use of calcium-containing phosphate binders and particularly the presence of severe CKD have been identified [8, 9].

Vascular calcification is now considered an active cell-mediated process similar to bone morphogenesis. Nevertheless, both origins of the mineralizing cell population and initiating stimuli remain controversial. One possible stimulus is bone morphogenetic protein 2 (BMP-2). It belongs to the transforming growth factor-β superfamily and acts by binding to its specific type II receptor followed by the activation of the type I receptor [10]. BMP-2 function can be inhibited by matrix Gla protein (MGP) [11], but only by the active Gla-MGP [12]. MGP activation is vitamin K dependent which needs to be activated via gamma-carboxylation [13]. Vitamin K antagonists such as warfarin inhibit the recycling of vitamin K, thereby limiting the activation of MGP. [14]. Vitamin K antagonist usage appears to be an independent risk factor for the development of CUA [15]. If BMP-2 is not inhibited by MGP, the BMP-2 downstream signalling cascade leads to an increased expression of the key osteogenic transcription factor Runx2 [16]. Runx2 controls the expression of matrix proteins of osteoblastic differentiation such as osteopontin and collagen I [17]. Sclerostin, the glycoprotein product of the SOST gene, is expressed by mature osteocytes and also antagonizes the effect of BMP-2 indirectly via inhibition of the Wnt/β-catenin signalling pathway [18, 19].

CUA can be regarded as a particular subtype of vascular calcification. It has been hypothesized that CUA is, similar to vascular calcification in large arteries, an active process with osteoblast-like cells contributing to calcification [20]. Thus, in calcified skin lesions of CUA patients, increased expression of osteopontin, suggesting an osteogenic process, was observed [20]. Besides osteogenesis, an altered extracellular matrix (ECM) with increased expression of matrix proteins such as collagen I, osteopontin and fibronectin is important in calcification processes [21, 22]. Clinically, hardening of the soft tissue with a dense, plate-like aspect is a frequent early finding in CUA patients, which proceeds later to ulcerations. And similarly, once soft-tissue ulcerations have developed, these lesions are often surrounded by areas of tissue induration typically palpable as leather-like rigidifications of skin areas in association with calcified and necrotic lesions [1].

Our study aimed to gain further insights into mechanistic aspects of CUA development with a special focus on the interplay between altered ECM remodelling, osteogenesis, ectopic calcification and vascular obstruction finally leading to tissue ulcerations in patients with CUA. We performed a systematic, detailed analysis of calcified skin specimens in seven CUA patients by histology, immunohistochemistry, electron microscopy, electron dispersive X-ray analysis (EDX) and quantitative real-time RT-PCR (Qt-RT-PCR). We particularly investigated markers involved in osteogenesis such as BMP2, Runx2, sclerostin and key proteins of the ECM such as collagen I, IV, fibronectin, osteopontin, MGP and laminin.

MATERIALS AND METHODS

CUA Patients

Based on data collected in our calciphylaxis registry initiative (International Collaborative Calciphylaxis Network), we retrospectively identified all patients who were treated for calciphylaxis in the University Hospital Aachen since 2006 and from whom tissue samples were available (n = 7; two males/five females, age 50 ± 19 years, range 21–77). We examined skin specimens obtained from consented clinical autopsies (n = 2) and operative resections (n = 5) of these seven CUA patients. The diagnosis of CUA of all patients was based on the clinical picture and confirmed by histological analysis of one or more skin sections. We used two different patient groups as controls: (i) patients without CUA and without CKD (non-CKD controls) and (ii) dialysis patients without CUA (dialysis controls). (i) As non-CKD controls, we matched skin specimens of seven non-CKD, non-CUA patients for age, gender, the presence or the absence of CKD (non-CKD controls) and (ii) dialysis patients without CUA (dialysis controls). (ii) As non-CKD controls, we matched skin specimens of seven non-CKD, non-CUA patients for age, gender, the presence or the absence of obesity and diabetes (n = 2 men, age 49 ± 18 years, range 25–76; diabetes in n = 4, obesity in n = 4). Specimens from non-CKD controls were obtained during consented clinical autopsies (n = 2), and by surgical skin resections in n = 5. The cause of death in all autopsy patients was septicemia. In autopsy cases, the skin samples were always resected within 8 h after death, and significant signs of autolysis were excluded. Due to our experience, early sampling of tissues of deceased subjects does not reduce the reliability of immuno-histochemical or EDX analysis and is also routinely applied for clinical autopsies. Only two autopsy cases were included into the PCR analysis (one in the CUA group and one in the non-CKD control group. (ii) As dialysis controls, we included skin samples obtained from dialysis patients without CUA (three males/four females, age 54 ± 10, range 45–71, diabetes in n = 4, obesity in n = 3, time on dialysis 4.2 ± 3.4 years). Specimens of dialysis control patients were obtained from
two lower leg amputations and from five surgical resections of healthy skin after skin cancer (melanoma). All patients and controls were of Caucasian ethnicity. Data about the medical history and medications were obtained by retrospective chart review and obtained from the above-mentioned calciphylaxis registry (www.calciphylaxie.de). The study was approved by the ethical committee of the RWTH Aachen University Hospital and carried out according to the principles of the Declaration of Helsinki.

Histomorphological analysis

For histological and immunohistochemical analyses, skin specimens were fixed in 3.7% formaldehyde for 24 h. Skin specimens were paraffin-embedded, cut with a rotating microtome at 3 µm thickness (Leica) and stained according to routine histology protocols. Immunohistochemical analysis was performed in an autostainer (DAKO cytomation) using primary antibodies specific for fibronectin (mouse monoclonal, 1:200; Sigma, Hamburg, Germany), collagen I (mouse monoclonal, 1:2000; Sigma), collagen IV (mouse monoclonal, 1:250; Sigma), laminin (mouse monoclonal, 1:1000; Sigma), CD68 (mouse monoclonal, 1:400; DAKO, Hamburg, Germany), CD31 (mouse monoclonal, 1:500; DAKO), BMP-2 (rabbit polyclonal, 1:200; Abcam, Cambridge, UK), Gluand Gla-MGP (mouse monoclonal, 1:200; Vascular Products, Maastricht, the Netherlands) and osteopontin (mouse monoclonal, 1:3000; Santa Cruz Biotechnology, Heidelberg, Germany) as described before [23, 24]. The immunostaining for sclerostin was performed manually. After blocking the sections for endogenous avidin/biotin (Avidin-Biotin Blocking Kit, Vector Laboratories Burlingame, CA, USA) and peroxidase activity (3% H₂O₂), slides were immediately incubated with a goat polyclonal anti-sclerostin antibody (1:200, R&D Systems; Minneapolis, MN, USA). Biotinylated horse monoclonal anti-goat antibody (Vector Laboratories) was used as a secondary antibody. Detection was realized using the Vectastin ABC Kit (Vector Laboratories).

Semi-quantitative immunohistochemical analysis

Protein expression and von Kossa staining were quantified by two blinded investigators (R.K. and R.K.S.) using a semi-quantitative scoring system (0, no expression; 1, weak expression; 2, moderate expression; 3, strong expression; 4, very strong expression) in high-power fields (×200 magnification) as described before [25, 26]. The number of small arteries and arterioles with CD31⁺ endoluminal debris was estimated by counting all arteries and arterioles containing endoluminal CD31⁺ cells in one section (magnification ×200) and dividing by all arteries and arterioles in the corresponding section.

Scanning electron microscopy

Tissue sections were fixed in 3% glutaraldehyde for at least 24 h, rinsed with sodium phosphate buffer (0.2 M, pH 7.39, MERCK, Darmstadt, Germany) and dehydrated by incubating consecutively in ascending acetone series (30, 50, 70 and 90%) with a final incubation in 100% acetone for 10 min. The tissue sections were critical-point dried in liquid CO₂. Samples were analysed using an environmental scanning electron microscope (ESEM XL 30 FEG, FEI, PHILIPS, Eindhoven, the Netherlands) in a high vacuum environment. EDX analysis was performed using an EDAX Falcon Genesis Spectrometer 5.21 energy-dispersive X-ray spectroscopy system with an ultrathin window liquid nitrogen cooled Si(Li) X-ray detector (EDAX Inc., Mahwah, NJ, USA).

Real-time reverse transcriptase polymerase chain reaction

Total RNA was isolated from the formalin-fixed, paraffin-embedded skin specimens using the RNeasy FFPE Kit (Qiagen, Hilden, Germany) according to the manufacturer instructions. We achieved an isolation of RNA feasible for real-time PCR from only four CUA patients (patient No. 1, 3, 5, 6) due to the origin of RNA from formalin-fixed paraffin-embedded sections. We compared gene expression of these four patients with gene expression of n = 4 non-CUA non-CKD controls and n = 4 non-CUA dialysis controls. The RNA concentration was determined by measuring absorbance at 260 nm (Nanodrop, Thermo Scientific, Wilmington, USA). One microgram of RNA was reverse transcribed using the high capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, 7300 Real-Time PCR System, Foster City, CA, USA). Quantitative PCR reactions were carried out with Power SYBR Green PCR Master Mix (Applied Biosystems). For each sample, 1.2 µL of cDNA was added as a template in PCR reactions. Amplification was monitored with the ABI Prism 7300 (Applied Biosystems). The expression of genes of interest was normalized against the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase in all samples. The cDNA of the skin sections from non-CUA non-CKD control patients was used as a relative standard for the genes of interest and set as one for all genes. Relative gene expression was analysed with the 2⁻ΔΔCt method. Primers are listed in Supplementary data, Table S1.

Statistical analysis

Data are presented as mean ± standard deviation (SD). After testing for normal distribution, one-way analysis of variance with the post hoc Scheffé procedure (PCR data) or Kruskall–Wallis test with post hoc Dunis multiple comparison (histological scoring) was used where appropriate. Statistical significance was defined as P < 0.05. Analyses were performed using PASW Statistic 18.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0c (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

Clinical results

Clinical data of the patients are presented in Table 1. All calciphylaxis patients were admitted to the University Hospital Aachen due to complicated CUA, and in all patients CUA was associated with chronic or severe acute kidney dysfunction: five patients were chronic haemodialysis (HD) patients, one patient suffered acute renal failure before CUA was diagnosed and one patient had CKD stage III. Six patients were
of Caucasian and one was of Asian ethnicity. Four patients were receiving long-term anti-coagulation with coumadin, four patients were substituted with oral vitamin D3, four patients were receiving subcutaneous injections of erythropoietin, one patient was receiving intravenous iron, six patients were receiving oral phosphorus binders (four sevelamer, one aluminium hydroxide, one lanthanum carbonate) and one patient was receiving cinacalcet. One haemodialysis patient had undergone parathyroidectomy. Laboratory data of the seven CUA patients are shown in Table 2. Three deaths occurred in HD CUA patients due to refractory septic shock.

The clinical picture of CUA was deep ulcerative proximal lesions in four patients, while three patients revealed distal calciphylaxis at the lower extremities.

Distribution of calcified lesions and upregulation of BMP-2, sclerostin and MGP
von Kossa staining revealed calcified areas in the subcutis of all CUA patients. Calcification was observed around adipocytes (Figure 1A), in the media of subcutaneous arterioles (Figure 1B, inset) and in connective tissue strands (Figure 1B) of the subcutis. Calcification was never observed in the dermis. To evaluate the hypothesis that active bone formation contributes to the process of subcutaneous calcification in CUA, we analysed the expression of BMP-2, its receptor bone morphogenic protein receptor 2 (BMPR-2), its downstream target gene Runx2 and its antagonists sclerostin and MGP in the skin of CUA patients. Immunohistochemistry showed areas positive for BMP-2 and sclerostin adjacent to calcified areas in the subcutis (Figure 1C–F) in all seven CUA patients. In contrast, the subcutis of controls (non-CKD and dialysis controls) was negative for sclerostin and BMP-2 (Table 3, Supplementary data, Figures S1 and S2). The results of the quantimental RT-PCR of four patients confirmed this observation at the mRNA level. BMP-2, sclerostin and Runx2 were significantly upregulated in the skin of CUA patients compared with the skin of both controls (Figure 1G). BMPR-2 was non-significantly upregulated in the skin of CUA patients compared with controls. Staining for inactive (uncarboxylated) Glu-MGP and active (carboxylated) Gla-MGP revealed an expression of both forms in CUA skin lesions (Figure 1H and Supplementary data, Figure S3). However, compared with the controls only the inactive Glu-MGP was significantly increased in the CUA skin (Table 3). We observed no differences in the expression of both MGP forms between coumadin-treated and non-treated CUA patients (data not shown).

CUA skin lesions exhibit extensive remodelling of the subcutaneous ECM
As fibrotic procalcific remodelling of the ECM supports calcification in heart valves and vessels [21, 22, 27], we hypothesized that a similar process is detectable in skin in durations of CUA patients which finally end in calcification. Semi-quantitative analysis of the protein expression in the subcutis revealed a significantly increased subcutaneous expression of collagen I, fibronectin, osteopontin and laminin in calcified skin specimens of CUA patients compared with non-CKD controls (Table 3). However, despite a trend towards upregulation of all analysed ECM proteins, semi-quantitative histological scoring revealed a significantly increased expression only for osteopontin and laminin in the CUA skin lesions compared with the dialysis controls (Figure 2 and Table 3). Osteopontin exhibited a comparable distribution pattern to von Kossa staining in CUA with a strong expression in the subcutis close to adipocytes and in connective tissue strands (Figure 2A–B). In contrast to the control skin (Figure 2D, inset and Supplementary data, Figure S4), the fine, reticular fibres surrounding adipocytes in CUA patients were broadened and strongly positive for fibronectin (Figure 2D).

In CUA, laminin and collagen I showed increased expression in association with adipocytes (Figure 2E and G), whereas collagen IV expression did not differ quantitatively from control skin, although the expression pattern was irregular around adipocytes in the subcutis of CUA patients, in terms of adiponecrosis (Figure 2F, Table 3). We compared the immunohistochemistry distribution pattern for ECM proteins to the von Kossa staining and observed an upregulation of fibronectin, collagen I and laminin even in areas that did not show overt calcification. Quantimental RT-PCR analysis supported the immunohistochemical findings regarding the extensive ECM remodelling process in CUA skin (Figure 2H), as osteopontin, fibronectin and laminin were significantly upregulated.

Calcification of the subcutaneous adipose tissue involves calcium hydroxyapatite formation
Scanning electron microscopy (SEM) confirmed calcified areas in the skin sections of all CUA patients (Figure 3). The most extensive calcification was detected in the media of subcutaneous arterioles (Figure 3B) and within the subcutaneous connective tissue (Figure 3F). Comparable with von Kossa staining, we detected calcifications around adipocytes (Figure 3E). Energy-dispersive X-ray spectroscopy (EDX) showed carbon, oxygen, phosphorus and calcium in all calcified regions (Figure 3D), with a molar ratio of calcium/phosphate of 1.68 ± 0.06 matching that of calcium hydroxyapatite mineral. Sodium was detected in five, iron in three, magnesium in two and silicium in one skin section of CUA patients (data not shown). Control spectra from skin sections of non-CKD controls showed carbon and oxygen (Figure 3C), but never calcium or phosphate.

Clogging of subcutaneous vessels by endothelial cells
In CUA lesions, lumina of small cutaneous vessels were often obstructed by cell debris (Figures 1B and 3B, inset). This observation had previously led to a ‘2-hit model’ where vascular calcification is followed by endoluminal obstruction, thrombus formation and tissue necrosis [1, 28]. Anti-CD31 staining revealed CD31+ endothelial cells (ECs) within the endoluminal debris in about 30% of the calcified cutaneous arteries and arterioles of calcified CUA skin sections (Figure 4A and B), while the normal CD31+ ECs...
layer of the intima was often destroyed in those vessels. In contrast, anti-CD31 staining of skin sections from non-CKD controls showed a regular CD31+ layer of intimal EC and never intraluminal cellular debris accumulation (Figure 4C). Furthermore, we observed histological signs of thrombosis in very small cutaneous vessels (diameter 15 μm) with remnants of CD31+ cells within the thrombus (Figure 4D–E).

**Haemosiderophages in the subcutis**

Iron has been discussed as a potential contributor to CUA development [29, 30] and, as noted above, we detected iron by EDX analysis in skin lesions of three of the seven CUA patients. In line with our EDX analysis, Prussian blue staining was positive in only these three skin sections. In two sections, only small Prussian blue-positive areas were observed in association with small subcutaneous vessels (Figure 5A, inset), while in the skin section of one CUA patient, we observed large Prussian blue-positive areas in the subcutaneous connective tissue (Figure 5A and B). SEM and EDX analyses revealed that the iron deposits were not closely correlated with the calcified areas, as we observed carbonate, oxygen, sodium, phosphorus and sulphate associated with the iron spectra, but not calcium (Figure 5C and D). Rather, anti-CD68 staining confirmed CD68+ macrophages within the iron deposits (Figure 5E and F), identifying them as haemosiderophages.

We observed no significant differences in any performed analyses between the proximal and distal form of CUA. This might be due to the small patient cohort.

### Table 1. Clinical characteristics of seven CUA patients

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (year)</th>
<th>Sex</th>
<th>Obesity</th>
<th>DM</th>
<th>Wound distribution</th>
<th>Cause of CKD/ESRD</th>
<th>RRT mode</th>
<th>Time on HD (years)</th>
<th>Outcome&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>F</td>
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<td>Yes</td>
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<td>Yes</td>
<td>Distal legs</td>
<td>Hypertensive</td>
<td>HD</td>
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<td>Alive</td>
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<td>No</td>
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<td>HD</td>
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<td>HD</td>
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<td>Expired</td>
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<td>M</td>
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<td>No</td>
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<td>CKD III diabetic</td>
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<td>–</td>
<td>Alive</td>
</tr>
</tbody>
</table>

<sup>a</sup>At discharge of hospital.

<sup>b</sup>Increase of creatinine up to 4.2 mg/dL in medical history.

### Table 2. Laboratory values of seven CUA patients upon admission

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Albumin (g/L)</th>
<th>Calcium (mmol/L)</th>
<th>Phosphorus (mmol/L)</th>
<th>AP (U/L)</th>
<th>PTH (ng/L)</th>
<th>Creatinine (mg/dL)</th>
<th>CRP&lt;sup&gt;a&lt;/sup&gt; (mg/L)</th>
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<td>2.7</td>
<td>115</td>
<td>896</td>
<td>12.2</td>
<td>38</td>
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<td>1.2</td>
<td>105</td>
<td>80</td>
<td>0.7</td>
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</table>

<sup>a</sup>Normal value <5 mg/L.

AP, alkaline phosphatase; CRP, C-reactive protein.
DISCUSSION

Once thought to be a passive process, vascular calcification has emerged as an active and tightly regulated cell-mediated process including matrix remodelling and deposition [16]. CUA can be regarded as a high-speed template for ectopic, vascular calcification processes with a rapid onset. Our current study points towards a number of yet unidentified issues in the pathogenesis of CUA at the tissue level: (i) we

FIGURE 1: Up-regulation of osteogenic markers in skin lesions of CUA patients. (A and B) von Kossa positive subcutaneous adipose tissue (A), connective tissue (B) and arteriole (B, inset). (C and D) BMP-2 staining and (E and F) sclerostin staining indicated an osteogenic process in the skin of CUA patients. We observed BMP2-positive areas in the dermis (C arrows) and the subcutaneous adipose tissue (D arrows) only in the calcified skin and subcutaneous areas of the CUA patients. Similarly, sclerostin positive areas were observed only in the subcutaneous adipose tissue of calcified skin in CUA patients (E and F arrows). The subcutis of control subjects was completely negative for these stainings (Supplementary data, Figures S1 and S2). (G) mRNA expression of BMP-2, sclerostin (SOST) and Runx2 was significantly enhanced in the skin lesions of CUA patients compared with the skin of both control groups (non-CKD controls = expression set as 1 for all genes). (H) Uncarboxylated inactive Glu-MGP expression was increased in the subcutaneous arterioles (arrow) and in the connective tissue surrounding adipocytes of the subcutis (H, insets, arrowheads) *P < 0.05 versus non-CKD controls, #P < 0.05 versus dialysis controls, values are expressed as mean ± SD. All scale bars 200 µm, insets 100 µm.
CUA skin lesions might reflect local vitamin K deficiency. However, we did not observe any differences between coumadin-treated and non-treated CUA patients in our small cohort.

Runx2 induction is an essential early step responsible for pre-osteoblast formation and plays an important role in enchondral ossification [38]. Further, Runx2 upregulates the expression of ECM proteins critical for osteogenesis such as osteopontin and collagen I [39]. This theory of a cascade in CUA development was supported by our second major finding, namely a significant upregulation of several ECM proteins critical for mineralization (osteopontin, fibronectin, collagen I and laminin) in skin lesions of CUA patients. ECM remodelling is important for osteogenesis and calcification in bone [38], heart valves [27] and vessels [21, 40]. Osteopontin expression in skin lesions of CUA was first described by Ahmed et al. [20]. These authors predominantly observed osteopontin-positive areas in the media of calcified cutaneous vessels. We additionally detected osteopontin in the calcified connective tissue surrounding adipocytes. As osteopontin is an important regulator of mineralization and blocks apatite crystal growth [41, 42], its increased expression in CUA might be a feedback mechanism to control uncontrolled growth of crystals in the CUA lesions. The most pronounced alteration of the ECM in the subcutis of CUA patients compared with control subjects was the strong expression of fibronectin even in non-calcified areas. Fibronectin might be a critical ECM protein for the calcification observed in the CUA lesions as it promotes the calcification of vascular smooth muscle cells in vitro [22]. Collagen I is another ECM protein in the CUA lesions and the major protein of bone that is essential for osteogenesis and also

### Table 3. Semiquantitative evaluation of protein expression and von Kossa staining in the subcutis of calcified CUA skin and control skin (from non-CKD patients and dialysis patients)

<table>
<thead>
<tr>
<th></th>
<th>Non-CKD controls (n = 7)</th>
<th>Dialysis controls (n = 7)</th>
<th>Non-CKD controls versus dialysis controls (P-value)</th>
<th>CUA (n = 7)</th>
<th>CUA versus non-CKD controls (P-value)</th>
<th>CUA versus dialysis controls (P-value)</th>
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<td>von Kossa</td>
<td>0</td>
<td>0.07 ± 0.19</td>
<td>ns</td>
<td>3.57 ± 0.35</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
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<tr>
<td>BMP-2</td>
<td>0</td>
<td>0.21 ± 0.27</td>
<td>ns</td>
<td>1.57 ± 0.19</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Sclerostin</td>
<td>0</td>
<td>0.14 ± 0.38</td>
<td>ns</td>
<td>1.79 ± 0.64</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Glu-MGP</td>
<td>0.17 ± 0.29</td>
<td>0.43 ± 0.53</td>
<td>ns</td>
<td>2.5 ± 0.71</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Gla-MGP</td>
<td>0.67 ± 0.29</td>
<td>1.07 ± 0.67</td>
<td>ns</td>
<td>1.57 ± 0.45</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>0</td>
<td>0.21 ± 0.27</td>
<td>ns</td>
<td>3.64 ± 0.48</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Collagen I</td>
<td>0.5 ± 0.5</td>
<td>1.14 ± 0.24</td>
<td>ns</td>
<td>1.86 ± 0.48</td>
<td>&lt;0.001</td>
<td>ns</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>1.79 ± 0.27</td>
<td>1.57 ± 0.45</td>
<td>ns</td>
<td>1.64 ± 0.38</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>0.86 ± 0.24</td>
<td>1.57 ± 0.45</td>
<td>ns</td>
<td>3.43 ± 0.45</td>
<td>&lt;0.001</td>
<td>ns</td>
</tr>
<tr>
<td>Laminin</td>
<td>0.57 ± 0.53</td>
<td>0.92 ± 0.19</td>
<td>ns</td>
<td>1.71 ± 0.39</td>
<td>&lt;0.01</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

*a Kruskall–Wallis test with post hoc Dunn’s multiple comparison test.

bMGP staining in healthy controls was only performed in n = 3 skin samples.

ns, non significant.
plays an important role in atherosclerosis and vascular calcification [21]. Collagen I expression was apparently upregulated in CUA skin specimen. Finally, we detected a significant upregulation of laminin in CUA skin, i.e. a key regulator of cell migration and adhesion [43]. This is in line with our previous finding of increased expression of laminin in calcified

FIGURE 2: ECM remodelling in the subcutis of CUA skin lesions. Immunohistochemistry demonstrated expression of various ECM proteins in CUA skin lesions in comparison with skin sections of dialysis controls (insets). (A and B) Osteopontin staining revealed positive areas with a similar distribution pattern as von Kossa staining (in Figure 1) around adipocytes (A and B arrows) and in the connective tissue strands of the subcutis (asterisk in A). (C and D) Fibronectin staining demonstrated strongly positive subcutaneous connective tissue with thickening of periadipocytic (arrows) and connective tissue (asterisk) fibres. (E) Laminin expression was slightly enhanced in CUA skin (F). Staining for collagen IV showed a more irregular distribution pattern in CUA subcutis (arrows) compared with skin of dialysis controls (inset). (G) Collagen I expression was increased in the subcutis of CUA patients (arrows). (H) mRNA expression of osteopontin (OPN) and fibronectin (FN) was significantly increased in the skin of CUA patients compared with both non-CKD and dialysis controls, whereas laminin (Lam) expression was only significantly increased in CUA versus non-CKD controls, collagen I (Col I) expression was non-significantly increased and collagen IV (Col IV) expression remained unchanged compared with skin of controls. Non-CKD controls = expression set as 1 for all genes; *P < 0.05 versus non-CKD controls, #P < 0.05 versus dialysis controls; values are expressed as mean ± SD; all scale bars 200 µm, except (F) and (F, inset) 100 µm.
vessels of dialysis patients [44]. In summary, we were able to detect a sophisticated concert of proteins in CUA lesions pointing towards a pro-calcifying ECM environment. This remodelling of the subcutaneous ECM in CUA may also contribute to the clinical finding of skin induration of CUA patients, which is a frequent early finding in areas that later progress to ulcerative lesions.

ECM remodelling in the so-called matrix maturation phase is associated with the expression of osteopontin which is thought to promote the deposition of mineral by regulating the amount and size of hydroxyapatite crystals formed [45]. This is consistent with our third major finding, namely the detection of hydroxyapatite within the calcified lesions of CUA patients. Calcium and phosphate were described within the calcified lesions of CUA patients before [5, 20], but we are the first to identify a molar ratio of calcium to phosphate of 1.6 matching that of hydroxyapatite mineral. This finding supports our hypothesis of active osteogenesis in CUA lesions. Hydroxyapatite is characterized by the incorporation of numerous foreign ions, which is consistent with our detection of small amounts of magnesium, sodium and the rare earth element silicium in the calcified CUA sections [46, 47]. However, our study cohort is too small to exclude the presence of any other calcium biominerals in CUA. Interestingly, besides hydroxyapatite, the presence of whitlockite was also reported in uraemic vascular calcification [48, 49].

Our fourth major novel finding was a significant upregulation of the osteocyte marker sclerostin in skin lesions of CUA.

**FIGURE 3:** Hydroxyapatite accumulation in the subcutis of CUA skin lesions. SEM demonstrated calcified areas in the subcutis (B, E and F) of CUA patients, whereas no calcification was observed in the skin of non-CKD controls (A). Adipose tissue (A) and subcutaneous vessels of non-CKD control (A, inset) did not show any calcification. In contrast, in CUA subcutis multiple calcified areas (B arrowhead, E and F) with strongly calcified subcutaneous arteries (B, inset) that partly contain endoluminal thrombosis (asterisk) were found. The calcification pattern surrounded the adipocytes (E arrows). EDX analysis only showed carbon and oxygen of the biological matrix in non-CKD skin (C), whereas calcium and phosphate were detected in the skin of CUA patients with a molar ratio of 1.6 matching that of hydroxyapatite mineral (D). The calcified areas in the subcutis of CUA patients were partially finely granular around adipocytes (E) and partially massive in the connective tissue (F). Scale bars 100 µm, except (F, inset) 20 µm.
patients. Sclerostin, first identified in 2001 [50], is an inhibitor of canonical Wnt signalling via binding to the frizzled-LRP5/6 membrane receptor complex leading to increased bone formation [51]. The regulation of sclerostin expression in osteocytes is not yet fully understood [52]. Calcitriol, glucocorticoids, TNF-\(\alpha\), BMP2, 4 and 6 are able to increase the sclerostin expression [52]. Increased serum values of sclerostin in ESRD patients compared with healthy control subjects have been described [53]. Recently, an upregulated sclerostin expression during vascular smooth muscle cell calcification \textit{in vitro} and in the calcified aorta of ectonucleotide pyrophosphate/phosphodiesterase 1-null (Enpp1\(^{-/-}\)) mice were observed [54]. Didangelos \textit{et al.} [55] detected sclerostin expression in intact human aortas obtained during aortic valve replacement. Overall, the role of sclerostin in uraemic bone disease and uraemic vascular calcification is incompletely understood. However, our immunohistochemistry and PCR data point towards an important role of sclerostin also in CUA. At this time-point, we are unable to establish any causality. However, regarding its role in the bone, the upregulation of sclerostin might be an antiregulatory process, as a last effort to prevent soft-tissue calcification.

The devastating clinical picture of the very severe form of calciphylaxis—the proximal ulcerative form [1]—is mainly caused by large ulcerative areas that often trigger infectious complications (septicaemia). It is likely that in CUA the primarily fibrotic and calcifying processes ultimately result in a fatal malperfusion syndrome. Indeed, we detected detached endoluminal CD31\(^+\) ECs and a destroyed endothelial cell layer of small- and medium-sized subcutaneous arteries in calcified CUA skin. Others previously also noted that the endothelium is ‘lifted off’ in calcified subcutaneous vessels of CUA skin sections [20]. Furthermore, we detected signs of total vascular occlusion in subcutaneous terminal vessels with thrombosis and remnants of CD31\(^+\) cells. Thus, a sequence may exist, whereby vascular calcification triggers endothelial damage finally leading to off-sheared ECs, which may be the nidus for thrombogenesis resulting in malperfusion and necrosis of the skin. The subcutaneous reduced blood flow might also promote the calcification of the subcutaneous soft tissue. In the context of thrombus formation, it is also interesting that BMPs (BMP-2/4) induce a proinflammatory endothelial phenotype and cause an induction of endothelial adhesion molecules in ECs with increased monocyte adhesion capability [31, 33, 56].

Prussian blue-positive areas have previously been reported in close spatial relation with vessels in CUA skin biopsies [29]. Iron deposition is considered a ‘challenging factor’ for CUA in analogy to what Hans Selye postulated in his animal models. Indeed, we detected iron via Prussian blue staining in the subcutis of three CUA patients, and EDX analysis confirmed the presence of iron by three typical iron peaks.

\textbf{FIGURE 4:} Obstruction of subcutaneous vessels by ECs in CUA. CD31 staining demonstrated a completely destroyed endothelial cell layer with clogging of small and medium subcutaneous arterioles (A and B arrows) by detached ECs in about 30% of the subcutaneous vessels in CUA patients in comparison with (C) regular endothelium in the subcutis of a non-CKD control (arrows CD31). We observed total endoluminal occlusion with residual CD31\(^+\) areas (arrow D and E) within thrombotic formations in smaller subcutaneous vessels (D and E). Scale bars A–C 50 \(\mu\)m, D and E 10 \(\mu\)m.
However, we do not consider iron as being a trigger factor for CUA. Given our observation that CD68 staining revealed an accumulation of macrophages exactly in these ‘iron-positive’ areas, the iron accumulation in the subcutis of CUA patients might be due to haemosiderophages. Interestingly, the one patient in our study who received i.v. iron therapy did not exhibit iron accumulation in the skin, which is in line with our hypothesis of secondary iron deposition due to haemorrhages. In summary, although the present cross-sectional study does not allow a clear causality to be established, our data point towards an active, cell-mediated, BMP-2-driven osteogenic process with extensive ECM remodelling and deposition of hydroxyapatite mineral in the subcutis of CUA patients. Although yet unproven, the present data confirm our clinical impression of a cascade starting with matrix remodelling, followed by calcification, endothelial damage and thrombus formation, and finally leading to luminal obstruction with tissue necrosis. Future research should focus on early causative factors for matrix remodelling and on the identification of driving forces for the progress of the above-postulated cascade in CUA.

SUPPLEMENTARY DATA

Supplementary data are available online at http://ndt.oxfordjournals.org.

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CONFLICT OF INTEREST STATEMENT

None declared.

REFERENCES


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