Ultrastructural Analysis of Vascular Calcifications in Uremia

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ABSTRACT

Accelerated intimal and medial calcification and sclerosis accompany the increased cardiovascular mortality of dialysis patients, but the pathomechanisms initiating microcalcifications of the media are largely unknown. In this study, we systematically investigated the ultrastructural properties of medial calcifications from patients with uremia. We collected iliac artery segments from 30 dialysis patients before kidney transplantation and studied them by radiography, microcomputed tomography, light microscopy, and transmission electron microscopy including electron energy loss spectrometry, energy dispersive spectroscopy, and electron diffraction. In addition, we performed synchrotron x-ray analyses and immunogold labeling to detect inhibitors of calcification. Von Kossa staining revealed calcification of 53% of the arteries. The diameter of these microcalcifications ranged from 20 to 500 nm, with a core-shell structure consisting of up to three layers (subshells). Many of the calcifications consisted of 2- to 10-nm nanocrystals and showed a hydroxyapatite and whitlockite crystalline structure and mineral phase. Immunogold labeling of calcification foci revealed the calcification inhibitors fetuin-A, osteopontin, and matrix gla protein. These observations suggest that uremic microcalcifications originate from nanocrystals, are chemically diverse, and intimately associate with proteinaceous inhibitors of calcification. Furthermore, considering the core-shell structure of the calcifications, apoptotic bodies or matrix vesicles may serve as a calcification nidus.


Cardiovascular mortality in patients with ESRD is dramatically increased when compared with the general population.¹ Cardiovascular calcifications are major predictors of mortality in patients with ESRD.²,³ Ectopic calcification is a tightly regulated process that results from an imbalance between proteinaceous and small-molecule inhibitors (e.g., fetuin, matrix gla protein, pyrophosphate) and inducers of mineralization (e.g., an elevated serum calcium-phosphate product).⁴,⁵ In cell culture models, hyperphosphatemia leads to a transformation of vascular smooth muscle cells to osteoblast-like cells, which then express bone-specific pro-
Thus, vascular wall calcifications in particular do not seem to result from passive precipitation of calcium and phosphate but rather involve active cellular processes.

So far, it is generally assumed that the mineral deposited in the vascular wall has the physicochemical properties of hydroxyapatite, the main mineral phase of bone; however, for the ultrastructure and systematic characterization of uremic cardiovascular calcifications, limited information is available in the literature. These previous studies used different tissues and yielded different results: Hydroxyapatite \([Ca_{10}(PO_4)_6(OH_2)]\)—as seen in the skeleton—was described as the sole mineral phase of uremic arterial calcifications in two studies, whereas another study found both brushite and hydroxyapatite in calcifications of stenotic arteriovenous fistulas.

In a rodent study of uremic calcification, whitlockite, a magnesium-containing crystal \([(Ca,Mg)_3(PO_4)_2]\), was detected in addition to hydroxyapatite.

Different mineral phases arising during uremic medial calcification may potentially be the result of different pathomechanisms ultimately affecting dissolution of the calcifications and the choice of therapy. We therefore performed a systematic ultrastructural analysis of uremic media calcifications in patients with ESRD.

### RESULTS

#### Prevalence and Predictors of Vascular Calcifications

Microcalcifications in the media of iliac arteries were detected by von Kossa staining in 53% of the samples. They were often diffusely dispersed in the tissue, but sometimes numerous cells were found nearby (Figure 1). The von Kossa and hematoxylin and eosin stains of our iliac arteries did not show intimal calcification or major atherosclerotic plaques. Moreover, samples with microcalcifications did not exhibit major histologic differences compared with samples without calcification (data not shown).

Neither the presence nor the degree of medial calcifications in iliac arteries was related to clinical or serum parameters (Table 1), but sample size limited the ability to detect differences. Of note, only one patient had a history of diabetes. Plain pelvic x-rays for the assessment of iliac calcifications in 19 of the 30 patients revealed that eight (73%) of 11 patients with a positive von Kossa stain for calcification exhibited calcifications on the plain pelvic x-ray, whereas only two (25%) of eight patients with a negative von Kossa stain had calcifications by x-ray \((P = 0.039)\). When comparing laboratory results of the 10 patients who had iliac calcifications on plain x-rays with the nine patients who did not have iliac calcifications, serum calcium \((2.56 \pm 0.16 \text{ versus } 2.39 \pm 0.19 \text{ mmol/L}; P = 0.049)\) and hemoglobin \((129 \pm 13 \text{ versus } 108 \pm 18 \text{ g/L}; P = 0.008)\) were increased, whereas the other parameters indicated in Table 1 did not differ significantly (data not shown).

#### Radiography and Microcomputed Tomography of Iliac Artery Calcification

Faxitron radiography and a three-dimensional reconstruction of mineral deposits in a uremic artery are shown in Figure 2. Calcification could be detected in three of four arteries and seemed extensive within certain regions of the artery, but closer examination revealed that all of these calcification sites were not continuous and contained smaller mineralized areas and microcalcifications, the majority of which seemed to be extracellular by microscopy of serial sections (Figures 1 and 3).

#### Electron Microscopic Morphology of Iliac Artery Calcifications

Microcalcifications were found in the vicinity of both healthy and damaged vascular smooth muscle cells. Moreover, these mineral deposits often occurred in areas rich in collagen and
proximately one third of the microcalcifications exhibited a core-shell layered structure (32%), some with more electron-dense calcification in the core than in the shell (22%), whereas most microcalcifications with core-shell layered structure lacked the electron-dense material in their core (78%). In some samples, a double-layered shell could be observed (Figure 4B). More rarely, a multi-shell structure with up to three subshells could be observed. When a core was present, the shells were arranged around a compact electron-dense spherical particle in its center. The spherulitic and layered nature was verified by a three-dimensional reconstruction from a tilt series (Figure 4E). Many of the calcified agglomerates consisted of spherical nanocrystals with grain sizes between 2 to 10 nm (Figure 4F).

**Chemical Composition of Iliac Artery Calcifications**

Using TEM techniques such as energy-dispersive x-ray spectroscopy (EDX) and electron energy loss spectroscopy (EELS), all spectra demonstrated the presence of the elements phosphorus, calcium, oxygen, and carbon in the calcified regions (Figure 5A). Control spectra taken from adjacent, noncalcified tissue always showed only amorphous carbon from the embedding material and/or biologic matrix, whereas different spectra were recorded from the electron-dense particles (Figure 5A).

EDX and EELS spectra of the electron-dense particles and vesicles identified elements consistent with hydroxyapatite as a chemical compound in microcalcifications of 10 samples, which was confirmed by crystallographic analysis using electron diffraction (Figure 5B). In addition to the TEM analyses, synchrotron radiation analysis was applied to six iliac arteries, which allowed for a more systematic scanning of the samples. Interestingly, synchrotron radiation analysis revealed the presence of whitlockite in addition to hydroxyapatite (Figure 5D).

### Table 1. Demographic data of patients undergoing kidney transplantation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>All Patients (n = 30)</th>
<th>No Iliac Artery Calcification (n = 14)</th>
<th>Iliac Artery Calcifications (n = 16)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years; mean ± SD)</td>
<td>49 ± 10</td>
<td>50 ± 9</td>
<td>49 ± 12</td>
<td>0.709</td>
</tr>
<tr>
<td>Male/female</td>
<td>17/13</td>
<td>7/7</td>
<td>10/6</td>
<td>0.491</td>
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<tr>
<td>Diabetes</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0.341</td>
</tr>
<tr>
<td>Hypertension</td>
<td>26</td>
<td>13</td>
<td>13</td>
<td>0.351</td>
</tr>
<tr>
<td>BMI (kg/m²; mean ± SD)</td>
<td>24.3 ± 3.2</td>
<td>24.5 ± 2.4</td>
<td>24.1 ± 3.9</td>
<td>0.692</td>
</tr>
<tr>
<td>Dialysis mode (HD/PD)</td>
<td>26/4</td>
<td>12/2</td>
<td>14/2</td>
<td>0.886</td>
</tr>
<tr>
<td>Dialysis vintage (years; mean ± SD)</td>
<td>5.9 ± 3.1</td>
<td>5.1 ± 3.1</td>
<td>6.7 ± 3.0</td>
<td>0.175</td>
</tr>
<tr>
<td>Hemoglobin (g/L; mean ± SD)</td>
<td>119 ± 17</td>
<td>119 ± 16</td>
<td>119 ± 18</td>
<td>0.733</td>
</tr>
<tr>
<td>Protein (g/L; mean ± SD)</td>
<td>68.7 ± 8.8</td>
<td>67.8 ± 8.3</td>
<td>69.4 ± 9.4</td>
<td>0.739</td>
</tr>
<tr>
<td>Calcium (mmol/L; mean ± SD)</td>
<td>2.47 ± 0.23</td>
<td>2.41 ± 0.22</td>
<td>2.51 ± 0.23</td>
<td>0.291</td>
</tr>
<tr>
<td>Phosphate (mmol/L; mean ± SD)</td>
<td>1.53 ± 0.58</td>
<td>1.51 ± 0.38</td>
<td>1.54 ± 0.71</td>
<td>0.759</td>
</tr>
<tr>
<td>Magnesium (mmol/L; mean ± SD)</td>
<td>0.91 ± 0.18</td>
<td>0.86 ± 0.16</td>
<td>0.96 ± 0.19</td>
<td>0.149</td>
</tr>
<tr>
<td>CRP (mg/L; mean ± SD)</td>
<td>14.2 ± 16.1</td>
<td>11.9 ± 14.9</td>
<td>16.0 ± 17.2</td>
<td>0.455</td>
</tr>
</tbody>
</table>

Calcification of the iliac artery was investigated by von Kossa stain. Blood was drawn before kidney transplantation. BMI, body mass index; CRP, C-reactive protein; HD, hemodialysis; PD, peritoneal dialysis.

**Figure 2.** Mineral deposits of variable size can be found in a uremic artery (longitudinal view) by use of x-ray imaging. (A) Radiograph showing a thin plate of mineralization (*) in the blood vessel wall, with occasional nearby mineralization foci (arrows). (B) Three-dimensional reconstruction of mineral deposits after microcomputed tomography of a uremic artery. A large mineralized area is apparent in addition to small mineralization foci (arrows). The insert shows a single x-ray microcomputed tomographic “slice” from this region used in the reconstructions.

vesicles (Figure 3), whereas an association with elastin fibrils or the inner elastic lamina could not be detected.

Transmission electron microscopy (TEM) revealed various morphologies of the microcalcifications (Figure 4). The diameter of the microcalcifications was mainly in the range of 20 to 500 nm, with only 12% of the microcalcifications exceeding 500 nm. Approxim
We could not analyze whether whitlockite was present only in certain types of calcification. Serum calcium, phosphate, and magnesium of the four patients with whitlockite were $2.42 \pm 0.29$, $0.99 \pm 0.39$, and $0.81 \pm 0.15$ mmol/L, respectively, whereas the mean serum concentration of these parameters of the two patients with hydroxyapatite were 2.25, 2.28, and 1.04 mmol/L, respectively.

In addition to analyses of iliac arteries, we investigated two brachial and two coronary arteries. Both brachial and coronary arteries showed a similar pattern of microcalcifications within their media. Using synchrotron radiation for diffraction analysis, the coronary arteries again showed both hydroxyapatite and whitlockite, whereas the brachial arteries contained only whitlockite.

Our second major finding was that some microcalcifications were composed of nanocrystals with a small size of 2 to 10 nm. To the best of our knowledge, such nano-sized calcifications have not yet been visualized in human media calcification. Becker et al. calculated a size of 20 to 25 nm for nanocrystals in atherosclerotic plaques. Recent data on calcification in an in vitro model indicated that spherical particles were composed of nanocrystalline needles.

In addition to ultrastructural analysis, we examined five uremic arteries (two iliac and three brachial arteries) for circulating and matrix mineral-binding proteins using immunoelectron microscopy. In all arteries investigated, immunogold labeling was consistently observed for fetuin-A, osteopontin, and matrix gla protein, showing a strong to moderate labeling of the microcalcifications (i.e., a close spatial relationship) but no labeling of the surrounding tissue for these proteins (Figure 6).

Our first major finding is that we discovered a core-shell layered structure in many of the microcalcifications and that some of these microcalcifications exhibited at least a double layer in their shell. In vitro calcification has been shown to be initiated by the release of membrane-bound matrix vesicles from vascular smooth muscle cells and/or by apoptotic bodies from dying cells acting as crystal nucleation points. Given the size of the core-shell structures in our study, the larger objects could represent calcification of apoptotic bodies and the smaller ones matrix vesicles, as has been described in a cell culture model of calcification. In nonuremic arteries (i.e., media of aorta and temporal arteries), objects with similar appearance to the microcalcifications detected in our study had been described. A recent study of human arteries showed that medial calcification was in part triggered by smooth muscle cell apoptosis. Whether the calcification process starts within the core or the shell structure still remains an open question. According to in vitro models, calcification can start both within vesicles and on the surface of vesicles. Alternative explanations are also possible, where a nidus of calcification (by whatever means) is followed by sequential bouts of growth and inhibition, leading to a layered structure.

Our second major finding was that some microcalcifications were composed of nanocrystals with a small size of 2 to 10 nm. To the best of our knowledge, such nano-sized calcifications have not yet been visualized in human media calcification. Becker et al. calculated a size of 20 to 25 nm for nanocrystals in atherosclerotic plaques. Recent data on calcification in an in vitro model indicated that spherical particles were composed of nanocrystalline needles.
the early stages of overt medial calcification. This could explain that does not progress and thus are not necessarily indicative of microcalcifications could also be a different form of calcification. A lamellar core-shell structure can be observed in many particles. (B through D) TEM of microcalcifications with a core-shell structure. A core was not present in all microcalcifications (D); however, when present (B and C), it consisted of more electron-dense material than the shell(s). (E) Three-dimensional reconstruction of the microcalcification shown in B with visualization of a solid core surrounded by an inner, less dense and an outer, more dense shell. (F) High-resolution TEM of a uremic vascular microcalcification. The microcalcifications consist of nanocrystals with a size of 2 to 10 nm.

Figure 4. The diameter of microcalcifications ranged from 20 to 500 nm and a core-shell layered structure could be noticed in approximately one third of the microcalcifications which suggest that apoptotic bodies or matrix vesicles may serve as a calcification nidus. Microcalcifications seem to originate from 2- to 10-nm nanocrystals. (A) TEM showing multiple microcalcifications with diameters between 20 and 500 nm. Microcalcifications show various morphologies. Of approximately 10 × 100 nm. In addition, it has been proposed that self-assembly of nano-apatite particles constitutes a subunit mechanism for biologic mineral crystals. Thus, our finding suggests that the nanocrystals play an early and essential role in the process of media calcification; however, a temporal sequence of microcrystals progressing to overt medial calcification cannot be proved by our cross-sectional study design. These microcalcifications could also be a different form of calcification that does not progress and thus are not necessarily indicative of the early stages of overt medial calcification. This could explain the discrepancy between the lack of calcification in elastin in this study and the calcification of elastin noted in other studies.

Our third major finding is the novel observation of whitlockite in human uremic arterial calcifications. Whitlockite is a magnesium-substituted crystal that has rarely been reported to be a component of human calcifications of the mesenteries, lymph nodes, dental calculus, or dystrophic gouty calcification of the aortic valve. In patients with uremia, whitlockite has been described as a preliminary stage in soft tissue calcifications but not in vascular calcifications. Most studies so far have exclusively detected hydroxyapatite or carbonated apatite in uremic vascular calcifications—except for one study, which reported brushite in arteriovenous fistulas. Whitlockite has been described only once as a chemical compound in the nonuremic human aorta; however, in this context, it has to be mentioned that hydroxyapatite rarely exists in its pure form in biologic systems but occurs often as carbonated apatite, which may also contain magnesium.

Our novel finding of whitlockite is at variance with two previous reports that described hydroxyapatite as the sole mineral phase present in human uremic vascular calcifications. Whereas in the study by Contiguglia et al., the vascular location of calcifications (i.e., intimal versus medial) was not reported, it is noteworthy that in the study by Becker et al., intimal but not medial uremic calcifications were investigated. Atherosclerosis and calcification of the arterial media (i.e., arteriosclerosis) may have overlapping mechanisms but differ in their pathogenesis. More important, in our study, whitlockite could be found only when using synchrotron radiation analysis but not with TEM techniques. With TEM techniques, only small parts of the tissue can be examined as a compromise for the available ultrahigh resolution. With synchrotron radiation analysis, however, because of the combination of micrometer resolution and short acquisition times (a few seconds), a more systematic scanning of the sample covering a larger area can be obtained, which is a major advantage of this technique—albeit at the cost of a lower resolution when compared with high-resolution TEM techniques. To the best of our knowledge, only Verberckmoes et al. applied synchrotron radiation analysis to examine vascular calcifications in rats with uremia, in which they also identified whitlockite as an important component of the mineral phase, particularly in vitamin D–treated animals. No studies using synchrotron radiation for diffraction analysis of human vascular calcification have been reported so far. This might be one of the reasons that in this study whitlockite was found as a novel compound in vascular calcification of human origin.

Our fourth major finding was the close spatial relationship between the microcalcifications and calcium-binding or calcification-inhibitory proteins. The proteins fetuin-A, matrix gla, and osteopontin are known calcification inhibitors, because their absence is associated with spontaneous calcifications in vivo and enhanced susceptibility to calcification in vitro. Indeed, all of these proteins have been shown to co-localize with uremic calcifications by immunohistologic methods. Until now, however, electron immunogold-labeling techniques for osteopontin and fetuin-A have been applied only to animal or in vitro models of
arterial calcification, respectively, but not to human arteries. Thus, our study provides further evidence that calcification inhibitors are indeed intricately involved in the process of calcification in humans. The localization of calcification inhibitors to areas of calcification is not yet completely understood. One possible explanation is an insufficient or exhausted defense mechanism against calcifications; however, other explanations are possible (e.g., removal of calcification complexes by vascular smooth muscle cells). Future studies are warranted for full understanding of the role of calcification inhibitors in the regulation of vascular calcification.

In conclusion, arterial microcalcifications in uremia seem to originate from nanocrystals. These uremic microcalcifications often exhibit a core-shell structure that may indicate a cellular origin. Calcification inhibitors were closely related to uremic calcifications. The novel observation of whitlockite within calcifications, in particular its potential clinical implications, requires further study.

Figure 5. Uremic arterial calcifications are chemically more diverse than previously thought with composition of hydroxyapatite and/or whitlockite. (A and B) Elemental and crystallographic analysis of microcalcifications using electron microscopy techniques. (A) EELS showing the spectrum of hydroxyapatite. (B) Electron diffraction pattern of hydroxyapatite. (C and D) Synchrotron radiation fluorescence and diffraction analysis. (C) Diffraction pattern for apatite (left) and whitlockite (right) standards. (D) The sample was scanned, and the results are depicted. The top panel shows the scan for the intensity of the calcium signal. The middle bottom panel shows the diffraction mapping, where the diffraction pattern of each point of the sample is represented in the scan on a one-to-one basis. The intensity of the signal is depicted by different colors. Both side panels show the diffraction pattern of the two points with the highest and second-highest calcium signal, respectively (as indicated by the white vertical line). The left spectrum reveals apatite as the chemical compound, whereas the right spectrum shows whitlockite.

Of all artery segments, one section was used for the von Kossa stain to assess whether microcalcifications were present. In 10 samples, we obtained sections of two parts (one at the beginning and one in the middle part of the arterial segment) and could confirm that von Kossa stain yielded the same results for these two parts (either only positive or only negative for calcification).

To assess the degree of calcification, we applied a semiquantitative score according to the study of Gross et al. Grade 0, no staining; grade 1, minimal positive staining; grade 2, positive staining involving up to 50% of the field of view; grade 3, positive staining involving >50%; grade 4, positive staining of all structures within the field of view.

For TEM techniques, tissue was fixed with glutaraldehyde and embedded in Epon (Serva, Heidelberg, Germany) or LR White acrylic resin (London Resin Company; Berkshire, UK) as described previously. In addition to iliac arteries, two pieces of brachial arteries were collected during arteriovenous fistula creation, and two coronary arteries were obtained from autopsy samples. Blood was drawn

CONCISE METHODS

Patients
We studied 30 dialysis patients who were undergoing renal transplantation. Patients were enrolled between March 2003 and March 2006. Characteristics of the patients are depicted in Table 1. None of the patients was on magnesium-containing phosphate binders. The study protocol adhered to the Declaration of Helsinki and was approved by the ethics committee of the Rheinisch-Westfälische Technische Hochschule Aachen, and each patient gave written informed consent.

Calcification Assessment
For 19 of the 30 patients, a plain pelvic x-ray for assessment of vascular calcification was performed. X-ray images were analyzed by one experienced physician, who was blinded to the patient’s condition.

Arteries and Biochemistry
Small pieces of iliac arteries (approximately 5 mm length), which normally would have been discarded, were obtained during renal transplantation. A standardized protocol was used for sample collection. Surgeons performed sampling at areas of the iliac artery that did not have any obvious macroscopic calcifications. Samples were fixed immediately in fixation buffer. For von Kossa staining (in paraffin sections) and synchrotron analysis, tissue was fixed in methacarn solution and embedded in paraffin. Sections were then stained using the von Kossa method and counterstained with hematoxylin and eosin as described previously.
at admission to the hospital before kidney transplantation. Biochemical analysis was performed by standard laboratory procedure using an automated analyzer.

Radiography and Microcomputed Tomography
High-resolution radiographic images of four artery segments were taken under identical conditions by means of a Faxitron Model MX-20 (Faxitron x-ray Corp., Wheeling, IL). Digital images were recorded at 26 kV and 0.3 mA over a 5-second exposure. For visualization of arterial calcifications in three dimensions, an x-ray microcomputed tomograph (SkyScan model 1072, Kontich, Belgium) was used to scan the arteries. The x-ray source was operated at maximum power (45 KeV) and at 222 m. Images were captured using a 12-bit cooled charge-coupled device camera (1024 × 1024 pixels) coupled by a fiber-optic taper to the scintillator. Using a rotation step of 0.9°, total scanning time was 35 minutes for each rotated sample. Sections were reconstructed using Skyscan tomography software (3D-Creator) based on a triangular surface rendering a three-dimensional distribution of the calcified tissue.

Ultrastructural Colloidal Gold Immunocytochemistry
The antibodies used for the immunocytochemistry included polyclonal anti-human fetuin-A (courtesy of Dr. W. Jahnen-Dechent, Aachen, Germany), polyclonal anti-mouse osteopontin (R&D Systems, Minneapolis, MN), and polyclonal anti-mouse matrix Gla protein (courtesy of Dr. L.W. Fisher, Bethesda, MD). Grid-mounted, LR White tissue sections were processed for colloidal gold immunocytochemistry by incubation of the sections containing microcalcifications with primary antibody (1:10 dilution), after which immunolabeling patterns were visualized by incubation with protein A–colloidal gold complex (14-nm gold particles; Dr. G. Posthuma, University of Utrecht, Utrecht, Netherlands), followed by conventional staining with uranyl acetate and lead citrate, as described previously.

Synchrotron Analysis
For synchrotron radiation μ-analysis (i.e., x-ray fluorescence and diffraction at the European Synchrotron Radiation Facility in Grenoble, France), we used unstained (and undecalcified) 10-μm-thick sections with both microcalcifications and areas of more dense calcification sequential to those used for the von Kossa staining as described already. X-ray fluorescence for calcium enabled localization of calcifications and indicated regions of interest to be further investigated by x-ray microdiffraction. Synthetic hydroxyapatite [Ca_{10}(PO_4)_6(OH)_{2}] and whitlockite [(Ca,Mg)_3(PO_4)_2] embedded in paraffin served as positive controls. A two-dimensional 135-mm MAR charge-coupled device–based diffraction camera (MarResearch, Norderstedt, Germany) was used to capture the diffraction patterns. A microfocus monochromatic x-ray beam (2 × 7 μm) with an energy of 14.4 keV was used to scan the samples. X-ray fluorescence mappings for calcium indicated calcified regions in the artery sections. Several line x-ray fluorescence and diffraction scans of adequate length (30 to 100 μm; 2-μm steps) were recorded per sample. Spectra of investigated samples were compared with synthetic mineral controls.
Statistical Analysis
Continuous variables were summarized by means and corresponding SD. Comparisons of the values of continuous variables between two groups and among three groups were made using an unpaired t test and ANOVA, respectively. Categorical variables were summarized by relative frequencies. \( \chi^2 \) test was used for investigating associations between various categorical variables.

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DISCLOSURES
None.

REFERENCES