Association between self-replicating calcifying nanoparticles and aortic stenosis: a possible link to valve calcification

Miguel A. Bratos-Pérez¹, Pedro L. Sánchez²*, Susana García de Cruz³, Eduardo Villacorta³, Igor F. Palacios⁴, José M. Fernández-Fernández¹, Salvatore Di Stefano³, Antonio Orduña-Domingo¹, Yolanda Carrascal³, Pedro Mota³, Cándido Martín-Luengo⁵, Javier Bermejo², José A. San Roman³, Antonio Rodríguez-Torres¹, and Francisco Fernández-Avilés² on behalf of Grupo AORTICA (Grupo de Estudio de la Estenosis Aórtica)

¹Servicio de Microbiología, Facultad de Medicina, Hospital Clínico Universitario, Valladolid, Spain; ²Servicio de Cardiología, Hospital General Universitario Gregorio Marañón, Doctor Esquero 46, 28007, Madrid, Spain; ³Instituto de Ciencias del Corazón (ICICOR), Hospital Clínico Universitario, Valladolid, Spain; ⁴Cardiac Unit, Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA; ⁵Servicio de Cardiología, Hospital Universitario de Salamanca, Spain

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Aims
Among various hypotheses proposed for pathological tissue calcification, recent evidence supports the possibility that self-replicating calcifying nanoparticles (CNPs) can contribute to such calcification. These CNPs have been detected and isolated from calcified human tissues, including blood vessels and kidney stones, and are referred to as nanobacteria. We evaluated calcific aortic valves for the presence of CNP.

Methods and results
Calcific aortic valves were obtained from 75 patients undergoing surgical valve replacement. The control group was formed by eight aortic valves corresponding to patients with heart transplants. In the microbiology laboratory, valves were screened for CNP using a 4–6 weeks specific culture method. The culture for CNP was positive in 48 of the 75 valves with aortic stenosis (64.0%) in comparison with zero of eight (0%) for the control group (P = 0.0005). The observation of cultures by way of scanning electron microscopy highlighted the resemblance in size and morphology of CNP.

Conclusion
Self-replicating calcific nanometer-scale particles, similar to those described as CNP from other calcific human tissues, can be cultured and visualized from calcific human aortic valves. This finding raises the question as to whether CNP contribute to the pathogenesis of the disease or whether they are only innocent bystanders.

Keywords
Nanobacteria • Calcifying nanoparticles • Aortic stenosis • Calcification

Introduction
Valvular aortic stenosis is currently the most frequent valvulopathy in adults in developed countries, and because of the continuous increase in life expectancy it is expected that its incidence will continue to rise, fundamentally at the expense of the ‘degenerative’ form.¹² Prevalence of this illness oscillates between 2 and 7% in adults over the age of 65,³ affecting more men than women, and representing the most frequent cause for valve replacement.

During the last few years, a diversity of studies, both retrospective and prospective, have suggested that common pathogenic mechanisms exist between degenerative aortic stenosis and coronary arteriosclerosis. Inflammatory processes are thought to be the underlying basis of these mechanisms, which are similar in aortic valves and in atheromatous plaques.⁴–⁷ Nonetheless, inflammation per se or as an isolated entity does not explain the presence of calcification and the progress to severe aortic stenosis, given that only 50% of adults with severe aortic stenosis suffer significant...
coronary disease and most patients with coronary illness do not suffer aortic stenosis.8

Among various hypotheses proposed to pathological tissue calcification, recent evidence supports the possibility that self-replicating calcifying nanoparticles (CNPs) can contribute to such calcification.7 These CNPs, referred to as nanobacteria, have the capacity themselves to precipitate calcium in the shape of apatite crystals at physiological calcium and phosphate concentrations.10,11 CNPs have been isolated from kidney stones and urine of patients with renal lithiasis,10,12 from renal fluid taken from patients with polycystic kidneys,13 from the biliary tract in patients with cholecystitis,14 from inclusions of psammoma in ovarian cancers,15 peripheral blood from healthy subjects,13 and from blood products.10,16,17 In addition to the association between CNP and stones, it has been suggested that these CNPs are strongly associated with diseases characterized by dystrophic calcification. These associations have been observed with electronic microscopy in carotids, aortic aneurysm, and calcified cardiac valves.16–20 and have been isolated from calcified cardiac arteries and valves,19 as well as from atheromatous plaques.21 However, relatively small size of these studies have limited the power for these associations.

To test the hypothesis that aortic valve calcification might be caused, in part, by these nano(bacteria) particles, we evaluate human calcific aortic valves for the presence of CNPs.

Methods

Clinical material

Calcific aortic valves were obtained from 75 patients undergoing valve replacement because of symptomatic severe aortic stenosis. Valves were obtained in a consecutive manner between January 2004 to July 2005. All patients had isolated severe aortic stenosis, as those with no severe aortic stenosis, more than mild aortic regurgitation, or mitral valve disease were excluded (n = 37). Thirteen valves obtained from patients initially included in the study did not undergo further measurements principally because of the unavailability of the microbiology laboratory to process the valve tissue. The control group was formed by eight non-stenotic aortic valves corresponding to patients with heart transplants, collected at the moment of explantation of recipient’s heart. In the control group, valves with either early sclerotic changes visualized by echo or with any visible morphological changes, during surgery, were excluded from the control population (n = 1). The study protocol was approved by the Ethics Committee and by the Hospital’s Investigation Committee. No patient, initially assessed for inclusion into the study, refused to enter the study.

Culture of nanosized particles

The valves were divided into two parts: one of which was frozen at −80 °C for future analysis and the other part was crushed in a sterile glass mortar. It was demineralized by adding 1 M HCl, which was subsequently neutralized with 1 M NaOH. The resultant was filtered with a 0.22 μm pore filter and cultured in DMEM supplemented with gamma-irradiated foetal bovine serum at 37 °C, under an atmosphere with 5–10% of CO2. After 6–8 weeks of culture incubation, it was re-suspended with the help of sterile glass pearls and re-inoculated in new Roux flasks under the above-described conditions.

The culture result from various Roux flasks was collected for microscopic observation, both under optical microscope and electronic microscope. For this, a culture scraping was executed in each flask, and then centrifuged as described above, combining the different sediments and washing with distilled water. The sediment was observed under the microscope in phase contrast. Gram, Ziehl-Neelsen, acridine orange stain, and Hoescht 33258 stain method were applied.12 For observation under transmission electron microscopy (TEM), the sediment was fixed with glutaraldehyde and subsequently subjected to post-fixing with osmium, dehydrated with acetone, and included in resin. The blocks were subsequently cut, carved at a thickness of 300 Å, and mounted over a copper grid. The samples were visualized with electronic transmission microscope JEOL JEM-1200 EXII. For observation with scanning electron microscopy (SEM), the nanoparticles were first cultured over a glass slide during 8 weeks, following the method described above. Subsequently, the glass slide was washed-out with distilled water, then fixed with glutaraldehyde at 2.5% and subsequently dehydrated in acetone and contrasted with gold. Visualization was executed with a JEOL JSM-820 microscope. Energy-dispersive X-ray microanalysis was executed with this same equipment.

Statistical analysis

Data are presented as medians (with the 25th and 75th percentiles), counts, or proportions. For comparison of the categorical variables, χ2 test was employed and also Fisher’s exact test if necessary. The Mann-Whitney U-non-parametric test was used to compare the continuous variables. Two-sided nominal unadjusted P-values < 0.05 were considered statistically significant, except when variables related to positive CNP cultures where analysed that we assumed a P-level of significance corrected by the Bonferroni method for multiple comparisons of 0.003 (0.05/number of testing (17) = 0.003). Although the study follows a prospective design, formal power calculation was not performed before starting the study as we could not find any data in the literature. The statistical package SPSS ver. 12.0 was used for all these calculations.

Results

The culture for CNP was positive in 48 of the 75 valves with aortic stenosis (64.0%) in comparison to zero of eight (0%) for the control group (P = 0.0005; 95% confidence interval for the difference between proportions, 0.53–0.75). At 6–8 weeks of incubation, a whitish precipitate appeared at the bottom of the positive test tubes, finely granulated and firmly adherent to the plastic. The observation of this precipitate under phase contrast provided non-specific images and the Gram, Ziehl-Neelsen, acridine orange, and Hoescht 33258 stain methods were negative in all cases. The appearance of a deposit at the bottom of the test tube with similar characteristics was observed in the sub-cultures (Figure 1). These strains have been maintained at the laboratory since then, by way of subcultures every 8 weeks in DMEM. In the successive subcultures, the deposit increasingly appeared faster and in greater amount.

The observation of the sediment by way of TEM (Figure 2) showed clearly isolated pleomorphic particles with a size of 0.2–0.5 μm. The SEM analysis (Figure 3) indicated spherical particles of 0.2 μm grouped in clusters. Figure 4 shows the results of energy-dispersive X-ray microanalysis where calcium...
and phosphorus, fundamental chemical elements that form apatite crystals, were observed.

The demographic, clinical, and echocardiography characteristics of the population with aortic stenosis and control groups are outlined in Table 1. The median age of these patients was 71 years; approximately half of the patients had a history of hypertension or dyslipaemia and one out of every four patients was diabetic or had a smoking habit. Prevalence of significant angiography coronary disease was 40%.

With the objective of identifying clinical or echocardiographic variables making evident a demographic, clinical, or echocardiography variable that could predict the presence of CNPs in the valves under study, a univariate analysis was performed assuming a P-level of significance corrected by the Bonferroni method for multiple comparisons, stratifying the patients with aortic stenosis to those...
with positive or negative cultures (Table 1). Only a trend towards increased male sex (68.8% vs. 40.7%, \(P = 0.03\) (greater than the Bonferroni-corrected \(P\)-value of 0.003)) and no-treatment with statins (61.4% vs. 33.3%, \(P = 0.04\) (greater than the Bonferroni-corrected \(P\)-value of 0.003)) was noted in patients with positive cultures.

### Discussion

The main finding of the present study was to show the presence of self-replicating CNPs in calcified valves in patients with aortic valve stenosis. Furthermore, CNPs are present in a greater proportion in calcified valves with aortic stenosis in comparison with non-calcified valves that were used as controls.

A controversy exists in regard to the nature of CNPs.\(^9,22\) It remains unclear whether such particles are living microorganisms or merely particles as CNPs have a scarce number of proteins in comparison with other common bacteria and that the nucleic acid cannot be detected with standard procedures.\(^11,23,24\) Thus, biomineralization attributed to nanobacteria may be initiated by non-living macromolecules and transferred on ‘subcultures’ by self-propagating microcrystalline apatite.\(^11\) Nonetheless, currently, there is highly also suggestive biochemical and/or molecular evidence supporting that CNP could represent an infectious agent: they are able to grow (even for years in our experience); exert cytotoxic effects,\(^25\) and bind to mononuclear antibodies;\(^14,15,19,26,27\) there is evidence of DNA\(^10,15,19,27,28\) and incorporation of radiolabelled substrate,\(^14,19,21\) there is evidence of proteins in serum-free cultures;\(^10\) they are susceptible to DNA synthesis inhibitor antimicrobial drugs even with no reports of calcium chelation activities,\(^29\) and they fulfil all Koch’s postulates.\(^27,30,31\)

For the first time, this study shows that CNP could be causally related with aortic stenosis in humans. Prior studies may have been limited by small numbers of patients.\(^19,20\) We suggest that CNPs colonize the aortic valve, provoking an inflammatory response, resulting in valve calcification via two distinct mechanisms: directly given their capacity to precipitate calcium in the shape of apatite crystals at physiological calcium and phosphate concentrations and indirectly by activating the inflammatory pathways. CNPs are able to infect phagocytosing cells via receptor-mediated internalization\(^10,32\) and have been shown to exert cytotoxic effects on fibroblasts.\(^10\) Thus, whether CNPs themselves serve as the nucleus for crystal formation or simply able to lower the activation energy barrier and thus allow the precipitation and growth of crystals under much lower supersaturation conditions is yet to be determined.\(^22\)

The hypothesis that low-grade chronic or recurrent bacterial endocarditis with specific calcifiable bacteria is a cause of calcification of the aortic valves has been previously investigated in an animal model.\(^33\) The results of this study suggested that recurrent low-grade endocarditis from calcifying oral bacteria, particularly when occurring with synergistic strains, may be one cause of calcific aortic stenosis. Of note, although the authors were not able to

### Table 1

<table>
<thead>
<tr>
<th>Variables</th>
<th>Controls</th>
<th>Aortic stenosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Total</td>
</tr>
<tr>
<td>Age, year (yr)</td>
<td>61 (58–67)</td>
<td>71 (67–76)</td>
</tr>
<tr>
<td>Male sex</td>
<td>8 (100)</td>
<td>44 (58.7)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>3 (37.5)</td>
<td>43 (59.7)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>3 (37.5)</td>
<td>16 (23.2)</td>
</tr>
<tr>
<td>Dyslipemia</td>
<td>4 (50.0)</td>
<td>35 (49.3)</td>
</tr>
<tr>
<td>Coronary angiographic disease</td>
<td>6 (75.0)</td>
<td>30 (40.5)</td>
</tr>
<tr>
<td>Medical treatment</td>
<td></td>
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<tr>
<td>Antiaggregants</td>
<td>4 (50.0)</td>
<td>31 (45.6)</td>
</tr>
<tr>
<td>Betablockers</td>
<td>5 (62.5)</td>
<td>25 (36.8)</td>
</tr>
<tr>
<td>Calcium antagonists</td>
<td>1 (12.5)</td>
<td>11 (16.2)</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>3 (37.5)</td>
<td>22 (31.9)</td>
</tr>
<tr>
<td>Statins</td>
<td>4 (50.0)</td>
<td>33 (48.5)</td>
</tr>
<tr>
<td>Transvavular peak gradient, mmHg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aortic valve area, cm(^2)</td>
<td></td>
<td></td>
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<tr>
<td>Total cholesterol, mg/dL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucocyte count, (\times 10^9) L(^{-1})</td>
<td></td>
<td></td>
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<tr>
<td>Fibrinogen, mg/dL</td>
<td></td>
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</tbody>
</table>

Categorical variables are presented as proportions, \(n\) (%). Continuous variables are presented as medians (with the 25th and 75th percentiles).

CNP, calcifying nanoparticles, also referred to as nanobacteria; ACE, angiotensin-converting enzyme.
culture the organisms injected (Corynebacterium matruchotti and Streptococcus sanguis) from the specimens, they were able to visualize small electron-dense objects containing myriad black needle-shaped crystals consistent with the size, form, and density of CNP, although these investigators interpreted their observation to calcified microbes and their membranous debris. It is important to emphasize that CNPs grow better in the presence of other bacteria and bind host defence proteins opening new insights into CNP interactions that could involve changes in the normal microbial flora, promote bacterial infection and bacterial biofilm formation. CNPs have been identified in tooth pulp stone, saliva, and dental plaque and may enter the blood stream during normal daily trauma to mucosal surfaces attaching themselves to the aortic valve leaflets.

If CNPs play a pathogenic role in aortic stenosis, vaccination and/or antimicrobians could have a protective effect. Given the ageing of the population and increases in the prevalence of aortic stenosis, our study has major clinical implications. Our findings could contribute to improve current treatment of patients with degenerative aortic stenosis, which, on the other hand, is quite limited. All of this in concordance with prior studies that have led towards the beneficial impact of a combined treatment with EDTA (ethylenediaminetetraacetic acid disodium salt) and tetracyclines in the reduction of calcification at a vascular arterial level. CNPs are also a good model system to use in developing vaccines to alter the likely diverse pathways involved in tissue calcification.

Limitations

The present study does not provide any evidence that CNPs are neither living organisms nor bacteria as we could not detect the presence of DNA using the Hoechst 33258 standard procedure. In this sense, some groups have recently been able to detect DNA in CNPs modifying the Hoechst method by increasing the stain concentration and prolonging the time extension. Although our findings could suggest a possible association between these nanoparticles and aortic valve calcification, a definitive cause and effect relationship needs to be established. For example, it will be necessary to evaluate severity of calcification and disease progression in the absence, presence, and titer of CNP in humans. In this sense, Ertas et al. have recently supported and strengthened this association. They reported that mean titers of CNP antibodies were higher in individuals with aortic valve calcification than in controls. They found a significant correlation between CNP antibody titers and the aortic calcification grade. Furthermore, increasing titers of CNP antibody were also associated with increased severity of aortic valve stenosis. Finally, in the experimental setting, because of the lack of aortic stenosis animal models, it will be difficult to prove infection of a naive animal pre-treated by endothelial denudation of one carotid in comparison with intravenously inoculation with either diluent or Escherichia coli-derived lipopolysaccharide.

In conclusion, this is the first study to identify a possible relationship between valve colonization by CNPs and calcific aortic valve stenosis. These data implicate a possible link between the presence of CNPs and the development of calcification, which is the hallmark of ‘degenerative’ aortic stenosis. However, whether CNPs contribute to the pathogenesis of the disease or are only innocent bystanders need to be clarified in further studies.

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Conflict of interest: none declared.

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References
