Endocarditis associated with contamination of cardiovascular bioprostheses with *Mycobacterium chelonae*: a collaborative microbiological study

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Summary

Background ***Mycobacterium chelonae*** **is a rare cause of infective endocarditis that is difficult to diagnose and treat. After we found *M chelonae* in a series of patients, we aimed to investigate its role in cardiovascular prosthesis dysfunction and contamination of bioprostheses as a possible cause of infection.**

Methods In this collaborative microbiological study, we report on nine patients treated in three cardiovascular surgical departments in Germany, who were found to have *M chelonae* infection after receiving BioIntegral bioprostheses. We performed fluorescence in-situ hybridisation (FISH) combined with broad-range 16S rRNA gene amplification and sequencing (FISHseq) on samples of native cardiovascular tissue and explanted bioprosthetic material, as well as on 12 unused BioIntegral prostheses. We confirmed FISHseq findings with histological examination by staining for acid-fast bacilli, and *M chelonae* was differentiated from *M abscessus* by molecular techniques.

Findings Between Dec 1, 2020, and Feb 28, 2022, we identified *M chelonae* in BioIntegral bioprostheses from three initial patients treated in Berlin that were explanted following dysfunction or suspected endocarditis, visualising morphologically intact FISH-positive mycobacteria. Despite negative mycobacterial culture, we also detected *M chelonae* in all 12 unused BioIntegral prostheses. The competent authorities in the EU prompted an alert, leading to the identification of six additional patients between March 1, 2022, and July 31, 2023. To find other cases of *M chelonae* endocarditis, we reviewed the FISHseq results of 1237 cardiovascular samples that were analysed between Jan 1, 2015, and Aug 31, 2022, including 295 samples from 228 bioprostheses supplied by other manufacturers. *M chelonae* was only detected in six of 41 patients who had received BioIntegral products.

Interpretation Bioprostheses manufactured by BioIntegral Surgical might be colonised by *M chelonae*, which can lead to implant dysfunction. These infections are likely to be missed by conventional routine diagnostics and should be considered in patients with BioIntegral implants and suspected infection or dysfunction. Cases should be reported to public health and regulatory authorities. Routine safety testing of bioprostheses during manufacture should be reconsidered.

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Introduction

Non-tuberculous mycobacteria (NTM) are rare causative pathogens of infective endocarditis, which can be missed by routine culture techniques and are difficult to treat. Contamination of heater-cooler devices is recognised as a leading cause of NTM infections following cardiovascular surgery, and *Mycobacterium chimaera*, which is a slowly growing NTM species, has been detected as the main pathogen.[1,2]

However, the source and role of rapidly growing NTM in cardiac infections has been under debate over the past four decades. *M chelonae* was first associated with prosthetic valve endocarditis in 1975,[3] and has since been reported in Brazil (18 of 39 valves; 1999–2008),[4] France (five of 370 valves; 2010–13),[5] and Switzerland (one valve; 2021).[6] *M chelonae* DNA has been detected previously in unused valves supplied directly from manufacturers (eg, Hancock porcine xenograft valves; Hancock Laboratories, Anaheim, CA, USA) as described by Tyras and colleagues[7] and in cases where the manufacturer is not disclosed, as described by Strabelli and colleagues.[4] Nevertheless, to date, knowledge on potential sources and transmission routes remains elusive. Options include colonisation of donor animals (eg, pigs or cattle), contamination in the animal facility during tissue sampling,

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Research in context

Evidence before this study

Since 1975, *Mycobacterium chelonae* has been documented as a rare but serious cause of infective endocarditis, with high mortality. Bioprostheses of porcine or bovine origin were under suspicion to be contaminated with *M chelonae*. From 1975 to 2002, infective endocarditis caused by a *Mycobacterium fortuitum–M chelonae* complex was documented in 20 cases, which were not restricted to prosthetic valves. To identify publications reporting on cardiovascular infections involving *M chelonae*, we searched PubMed, without language restrictions, for studies published from the date of database inception to Feb 15, 2023, using the following search terms: (((chelonae) OR (chelonei))) AND ((heart) OR (endocarditis) OR (cardiovascular) OR (ventricular) OR (open heart surgery) OR (valve) OR (aorta) OR (prosthesis) OR (prostheses) OR (cardiac surgery) OR (biological prosthesis)). Our search resulted in a total of 91 peer-reviewed publications featuring case reports or series (n=53), original research articles (n=26), and reviews (n=12). We excluded publications not addressing cardiovascular infections (eg, orthopaedic devices, endoscopes or bronchoscopes, transplant recipients, ophthalmic infections, or wound infections) or those addressing cardiovascular implantable electronic devices or ventricular assist devices, resulting in ten case reports or series, six original research articles, and four reviews mentioning endocarditis caused by *M chelonae*.

To avoid missing any association between other non-tuberculous mycobacteria (NTM) and the contamination of bioprostheses, we expanded the literature search using the following search terms: (("mycobacteria other than tuberculosis") OR ("non-tuberculous mycobact*")) AND ((heart) OR (endocarditis) OR (cardiovascular) OR (ventricular) OR (open heart surgery) OR (valve) OR (aorta) OR (prosthesis) OR (prostheses) OR (cardiac surgery) OR (biological prosthesis)). We found 182 publications describing various NTM; given that only *M chelonae* was detected in our samples, we focused on this species only.

In 2010, the first systematic retrospective analysis of endocarditis caused by bioprosthetic valves contaminated with culture-negative NTM (1999–2008), in which 18 (46%) of 39 valves (13 patients) were contaminated with acid-fast bacilli, was published. Sequencing of five prostheses from the same manufacturer (not named) detected *M chelonae*. Despite cultures testing negative for NTM, acid-fast bacilli were detected in five of six unused valves from the same manufacturer. Similarly, in 2015, an association between the implantation of bioprosthetic valves and endocarditis caused by NTM was found. Nucleic acid amplification techniques of 370 valves detected *M chelonae* in five valves from four patients (2010–13). Again, all cultures remained negative for NTM. Likewise, in 2021, a single case of endocarditis caused by a bioprosthetic valve contaminated with *M chelonae* was detected using next-generation sequencing after all other methods had failed. Taken together, the suspicion that *M chelonae* causes prosthesis dysfunction through contaminated bioprosthetic valves has existed since 1977. Although individual reports and cohorts have been published, this theory has not yet been investigated because cases were difficult to diagnose and tracing back to the manufacturing process or source was not successful.

Added value of this study

Using a combination of molecular, microscopic, and histopathological techniques, we provide novel evidence that bioprosthetic heart valves supplied by a single manufacturer, BioIntegral Surgical, were contaminated by *M chelonae*. This study shows the in-situ clinical and histopathological consequences that heart valve prostheses contaminated with *M chelonae* can cause in patients, despite negative culture results. Using fluorescence in-situ hybridisation, we were able to show morphologically intact DNA and rRNA containing mycobacteria in cardiovascular samples and between the heart muscle cells of patients, leading to tissue disruption and an immune response with granulomas, pointing to dysfunction of the prostheses.

Implications of all the available evidence

Medical professionals need to be alerted to this source of infection, which is not detected by routine diagnostic measures but could seriously compromise patient safety. It is essential to consider *M chelonae* infection in patients with BioIntegral prostheses, in particular, given that this contamination cannot be restricted to specific lot numbers or production periods. Patients at risk currently living with BioIntegral prostheses should be monitored with appropriate awareness. Due to the difficult diagnosis of *M chelonae* with routine methods, advanced diagnostics should be used in affected patients, such as molecular diagnostics and mycobacterial culture.

The identification of *M chelonae* in unused grafts implies contamination during either the harvesting process in the animal facility or the bioprostheses production process. Future molecular investigations must identify whether a single bacterial strain or multiple sources can be assumed responsible for contamination. Standardisation bodies and competent authorities should review the current standards and regulatory requirements on medical devices that use tissues of animal origin to reflect these findings. Routine safety testing of bioprostheses during manufacturing should be reconsidered, including molecular techniques.

Based on notification of the cases from this study, the competent authorities in the EU prompted an alert in 2022. However, to our knowledge, there are currently no restrictions for the use of BioIntegral prostheses outside the EU.

or inoculation during manufacturing.[4,7,8] The clinical relevance of detecting mycobacterial DNA in prostheses remains unclear.

After we detected and visualised *Mycobacterium abscessus–chelonae* complex in three patients who had undergone cardiac surgery in Berlin, Germany, using fluorescence

in-situ hybridisation (FISH) combined with broad-range 16S rRNA gene amplification and sequencing (FISHseq) between Dec 1, 2020, and Feb 28, 2022, we noticed that all patients had a cardiovascular bioprosthesis from the same manufacturer (BioIntegral Surgical, Mississauga, ON, Canada). Therefore, we suspected contamination of these prostheses as a source of infection and raised an alert to the German Federal Institute for Drugs and Medical Devices. To confirm our suspicion, we formed an interdisciplinary collaborative team to investigate clinical specimens and unused BioIntegral bioprostheses with FISHseq, additional molecular techniques, microbiological cultures, and stains. Eight additional patients with *M chelonae* infection following receipt of a bioprosthesis from BioIntegral Surgical were diagnosed in Germany, France, and Switzerland.[6]

In this collaborative diagnostic effort, we aimed to investigate the in-situ histopathological evidence and clinical consequences of heart valve prostheses contaminated with *M chelonae*, despite negative culture results. Taken together, we detected *M chelonae* using different molecular and microscopic methods exclusively in prostheses from the same manufacturer.

Methods

Study design and participants

In this this collaborative microbiological study, we report on nine patients treated in three cardiovascular surgical departments in Germany, who were found to have *M chelonae* infection after receiving a BioIntegral bioprosthesis. Intraoperatively fixated cardiovascular samples (both native tissue and prosthetic materials) are routinely sent for FISHseq analysis by several heart centres in Germany according to each centre's diagnostic protocol for infective endocarditis and the 2023 Duke–International Society for Cardiovascular Infectious Diseases (ISCVID) criteria.[9,10] All cardiovascular samples submitted for FISHseq from the three heart centres in Germany were included in the retrospective review. A case was defined as a patient with detected NTM in a sample of explanted prosthetic material or native cardiovascular tissue. As the exposure criterion, open-heart surgery was documented. The timeline of events (Dec 1, 2020–July 31, 2023) is provided in the appendix (p 2).

This study was approved by the ethics committees of Charité–Universitätsmedizin Berlin (EA4/130/21), Technical University of Munich (2022-462-S-SR), and University Hospital Schleswig-Holstein (B 316/22). Given that all samples were submitted for FISHseq analysis for diagnostic purposes, the ethics committees waived informed consent from patients. All patients whose detailed medical history is described in this study provided written informed consent.

Procedures

Regarding FISHseq, clinical samples were fixed during surgery and unused prostheses were fixed in the laboratory (directly after opening the originally packed container under a laminar flow cabinet) in FISHopt (MoKi Analytics, Berlin, Germany; appendix pp 23–25). These samples, as well as control strains, were embedded in methacrylate and sectioned as described previously.[9,11] We performed FISH using pan-bacterial, NTM-specific, and *Streptococcus*-specific peptide nucleic acid (PNA) FISH probes, and 4′,6-diamidino-2-phenylindole (DAPI) nucleic acid stain (appendix pp 3, 13–22).

Consecutive sections of the identical methacrylate block that was used for FISH were used for DNA isolation, followed by pan-bacterial and mycobacteria-specific 16S rRNA gene PCR and sequencing.[9,12] Sequences were analysed with a commercial 16S rRNA gene database (SmartGene, Lausanne, Switzerland; appendix p 3).

For further identification, we performed the GenoType Mycobacterium common mycobacteria (CM) and GenoType NTM drug resistance (NTM-DR) line probe assays (Hain Lifescience, Nehren, Germany) and amplified and sequenced the mycobacteria-specific internal transcribed spacer region between the 16S and 23S rRNA gene (appendix pp 3–4).[13] We also performed the GenoType NTM-DR line probe assay to cover possible resistance mechanisms.

For next-generation sequencing, we extracted and purified genomic DNA using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany), and prepared an Illumina sequencing library using the QIAseq FX DNA Library Kit (Qiagen, Hilden, Germany). Sequencing was performed on an Illumina MiSeq platform (Illumina, San Diego, CA, USA; appendix p 4).

To detect acid-fast bacilli, we performed microscopic examination of native cardiovascular tissue with Auramine, Ziehl–Neelsen, and Kinyoun staining. Histological examination included haematoxylin and eosin staining and anti-CD68 immunohistochemistry to label macrophages (appendix p 4).

Standard microbiological cultures for infective endocarditis were performed routinely. For unused BioIntegral prostheses, and whenever material from clinical samples was available at the time of FISHseq diagnosis, mycobacterial cultures were initiated at 30°C and 37°C in a liquid medium (Mycobacteria Growth Indicator Tube, Becton Dickinson, Franklin Lakes, NJ, USA) and two solid media (Stonebrink and Löwenstein–Jensen, Artelt-Enclit, Borna, Germany) for at least 12 weeks (appendix pp 4–5).

See Online for appendix

Statistical analysis

To establish whether *M chelonae* was confined to a single manufacturer, hospital, or particular sample type, we performed a retrospective evaluation of all available FISHseq results of samples previously received from three different cardiac surgery centres across Germany (Munich Clinic Bogenhausen [01/2015-08/2022], German Heart Center Berlin [01/2017-08/2022], and University Hospital Schleswig-Holstein [10/2019-07/2022]). We retrospectively evaluated all FISHseq results performed between January, 2015, and August, 2022, for the diagnosis of

suspected cardiovascular infection, consisting of a total of 1237 consecutive cardiovascular samples from 971 patients.

We calculated odds ratios (ORs), 95% CIs, and p values according to Woolf's method with Haldane–Anscombe correction, with an additive correction term of 0·5 in each cell (appendix p 5). All analyses were performed in Stata (version 17.0).

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Between Dec 1, 2020, and Feb 28, 2022, *M chelonae* was detected in three patients whose prosthetic heart valves had been explanted due to signs of infection, dysfunction, or both. These three bioprostheses were supplied by the same manufacturer (BioIntegral Surgical) and had been implanted in the patients for between 12 days and 4 years (table 1). Following an alert from the authorities, samples of BioIntegral prosthetic material from three additional patients with signs of *M chelonae* infection and implant dysfunction were referred for FISHseq analysis between March 1 and Dec 31, 2022, and from three more patients after the end date of this study between Jan 1 and June 30, 2023 (table 1; appendix p 2). Detailed clinical information on patients 1 and 4 is provided in the appendix (p 7).

Regarding quality control for FISH, NTM were reliably detected by the PNA FISH probe and no unspecific FISH probe binding was observed in the panel of non-target strains (appendix pp 3, 13–14). PNA FISH of cross-sections of *M chelonae* colonies as positive controls showed layers with different signal intensity corresponding to different ribosome content, thus indicating activity of FISH-positive mycobacteria (appendix pp 15–16).

In samples of prosthetic material from patients 1–4, 8, and 9, FISHseq detected *M abscessus–chelonae* complex exclusively (appendix pp 8–10). Within patient tissues, morphologically intact, FISH-positive (thus ribosome-containing) mycobacteria were visualised (figures 1–3). In samples from patient 5, FISHseq showed extensive *S aureus* biofilms (appendix p 19) and only PCR specific to mycobacteria allowed sequencing of the *M abscessus–chelonae* complex. In samples from patients 6 and 7, microscopy showed rods typical for mycobacteria in DAPI that were PNA FISH-negative; however, PCR and sequencing showed *M abscessus–chelonae* complex.

Standard cultures of explanted prosthetic heart valves remained negative for NTM, but showed growth of other bacteria in patient 2 (*Enterococcus faecium*), patient 4 (*Staphylococcus epidermidis*), and patient 5 (*S aureus*). None of the specimens yielded growth of mycobacteria in a mycobacterial culture, which in most cases was not performed in a timely and specific manner. Auramine, Ziehl–Neelsen, or Kinyoun staining of methacrylate tissue sections confirmed the presence of acid-fast bacilli in six of the eight patients investigated (appendix pp 8–10), partly between patients' heart muscle cells in close proximity to sutures or prosthetic material (figures 2, 3; appendix pp 20–22).

In all samples except for those from patient 4, *M chelonae* DNA was confirmed in the German National Reference Center for Mycobacteria by at least two of the following molecular diagnostics methods: GenoType Mycobacterium CM line probe assay, GenoType NTM-DR line probe assay, or sequencing of the internal transcribed spacer region. In patient 4, NTM could only be differentiated as *M chelonae–immunogenum* by GenoType Mycobacterium CM line probe assay (appendix pp 9–10). In patient 3, next-generation sequencing confirmed *M chelonae* by BLAST search against the NCBI nucleotide database (average sequence identity 99·32%).

In patient 1, histological evaluation assessed semi-quantitatively by microscopy showed cellular granulation tissue at the interface between the prosthetic valve and native cardiovascular tissue, which consisted of loose connective tissue, capillaries, and an inflammatory infiltrate containing T cells (staining not shown) and numerous $CD68^+$ macrophages with epithelioid granuloma formation (figure 2). In an identical section of tissue stained with haematoxylin and eosin from patient 3, NTM were detected by FISH and were localised in an area where cardiomyocytes were replaced by fibrous tissue adjacent to suture material (figure 3A–G). Consecutive sections were stained with Kinyoun staining, which confirmed acid-fast bacilli between muscle cells (figure 3H–J). Overall, mycobacterial load was high in all patients except for patient 5, not only in valve tissue and along sutures but also in numerous small groups of bacteria in the patients' tissue (figures 1–3; appendix pp 17–18, 20–22).

In 12 unused BioIntegral prostheses, the GenoType Mycobacterium CM line probe assay and sequencing of the internal transcribed spacer gene region identified *M chelonae* DNA in all samples (appendix p 11). Additionally, for three of the valves that were investigated, the *rrs–rrl* gene analysis by GenoType NTM-DR line probe assay showed no evidence of resistance mutations. Auramine staining detected varying amounts of acid-fast bacilli (appendix pp 17–18). FISHseq proved morphologically intact NTM in all seven prosthetic valves that were investigated (appendix pp 17–18). No cultural growth was observed after 12 weeks of incubation at 30°C and 37°C.

To find other cases of *M chelonae* endocarditis and to establish if *M chelonae* was associated with a specific hospital, laboratory, or prosthesis manufacturer, we reviewed the FISHseq results of 1237 cardiovascular samples. *M chelonae* was detected in 11 samples from six patients who had all received BioIntegral prostheses. Throughout the study period, *M chelonae* was not detected in a further 60 samples from 35 BioIntegral prostheses, nor in 295 samples from 228 bioprostheses supplied by other manufacturers (table 2; appendix p 12).

Based on Woolf's method with Haldane–Anscombe correction, we obtained an estimated OR of 83·68 (95% CI

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age at time of surgery with first detection of *Mycobacterium chelonae*, years	40	27	56	47	44	65
Sex	Male	Female	Male	Male	Female	Male
Prosthesis implantation						
Reason for implantation	Infective endocarditis of native valve	Infective endocarditis of native valve	Infective endocarditis of prosthetic valve (transcatheter aortic valve replacement)	Type A aortic dissection	Infective endocarditis of native valve	Infective endocarditis of prosthetic valve
Years with implanted valve	2017–20	2021	2022	2022	2021–22	2021–22
Duration of implant	46 months and 3 days	3 months and 13 days	12 days	6 months and 12 days	14 months and 4 days	16 months and 24 days
Underlying disease or condition	None	Intravenous drug use, groin infection, central line-associated bloodstream infection, HCV	None	Familial thoracic aortic aneurysm and dissection (Gsell-Erdheim syndrome)	Intravenous drug use, HIV, HCV	None
Prosthesis type						
Name	No-React BioConduit	No-React BioMitral	No-React BioAortic	No-React BioConduit	No-React BioAortic	No-React BioConduit
Manufacturer	BioIntegral Surgical	BioIntegral Surgical	BioIntegral Surgical	BioIntegral Surgical	BioIntegral Surgical	BioIntegral Surgical
Prosthesis removal						
Reason for removal	Infective endocarditis of prosthetic valve	Infective endocarditis of prosthetic valve	Suspected continuous localised infection in tissue	Increased focal metabolic activity on PET-CT along prosthetic ascending aorta and suspected periprosthetic hyperinflammation along median sternotomy wound with possible connection between the prosthesis, periprosthetic infection, or acute rupture of an aneurysm	Infective endocarditis of prosthetic valve	Infective endocarditis of prosthetic valve
Signs of infection	Fever, shivering, and joint pain	None	None	Slightly elevated CRP	Acute infection, elevated CRP and white blood cell count	Stroke, atrioventricular block and left bundle branch block with pacemaker implantation, atrial fibrillation, no signs of acute infection
Clinical results	Abscess formation along graft (PET-CT)	Vegetation at graft and ventricular septum defect, recurrent pericardial effusion (transoesophageal echocardiogram)	Paravalvular leakage and aortic regurgitation with acute decompensation	Inconspicuous	Septic shock with multi-organ insufficiency, stroke (hemiparesis and aphasia)	Paravalvular and para-aortal abscess formation with compression of coronary artery and aortic regurgitation due to dehiscence of aortic valve
Microbiological results						
Blood culture results within 4 weeks before surgery (number of positive cultures/number of total bottles)	*Clostridium baratii* (1/34, time to positivity 92 h); *Enterococcus faecalis* (1/34, time to positivity 114 h)	*Enterococcus faecium* (12/25); *Candida glabrata* (2/25)	*Streptococcus gallolyticus pasteurianus* (9/28); *Staphylococcus lugdunensis* (1/28)	*Staphylococcus epidermidis* (6/6)	*Staphylococcus aureus* (1/4)	Negative (14)
Tissue culture species	Negative	*E faecium*	Negative	*S epidermidis*	*S aureus*	Negative
Mycobacterial culture	Not done	Not done	Negative, started delayed, only after FISHseq result	Negative	Negative	Negative
Molecular results of tissue graft	*M chelonae*, no indication of other species	*M chelonae*, no indication of other species	*M chelonae*, no indication of other species	*M chelonae*, no indication for other species	*S aureus* and *M chelonae*	*M chelonae*
Clinical outcomes	Continuous signs of infective endocarditis on PET-CT, receipt of anti-mycobacterial therapy	Infective endocarditis with *Arcanobacterium haemolyticum* and *Anaerococcus* after 1 year, tricuspid valve replacement	Death due to clinical aggravation with MODS	6 weeks of antibiotic treatment for *S epidermidis* combined with antibiotic treatment for *M chelonae* (ongoing)	6 weeks antibiotic treatment for *S aureus*, no anti-mycobacterial therapy, close follow-up by infectious disease specialists	Death due to MODS and disseminated intravascular coagulopathy

HCV=hepatitis C virus. CRP=C-reactive protein. FISHseq=fluorescence in-situ hybridisation combined with broad-range 16S rRNA gene PCR and sequencing. MODS=multiple organ dysfunction syndrome.

Table 1: **Patient characteristics**

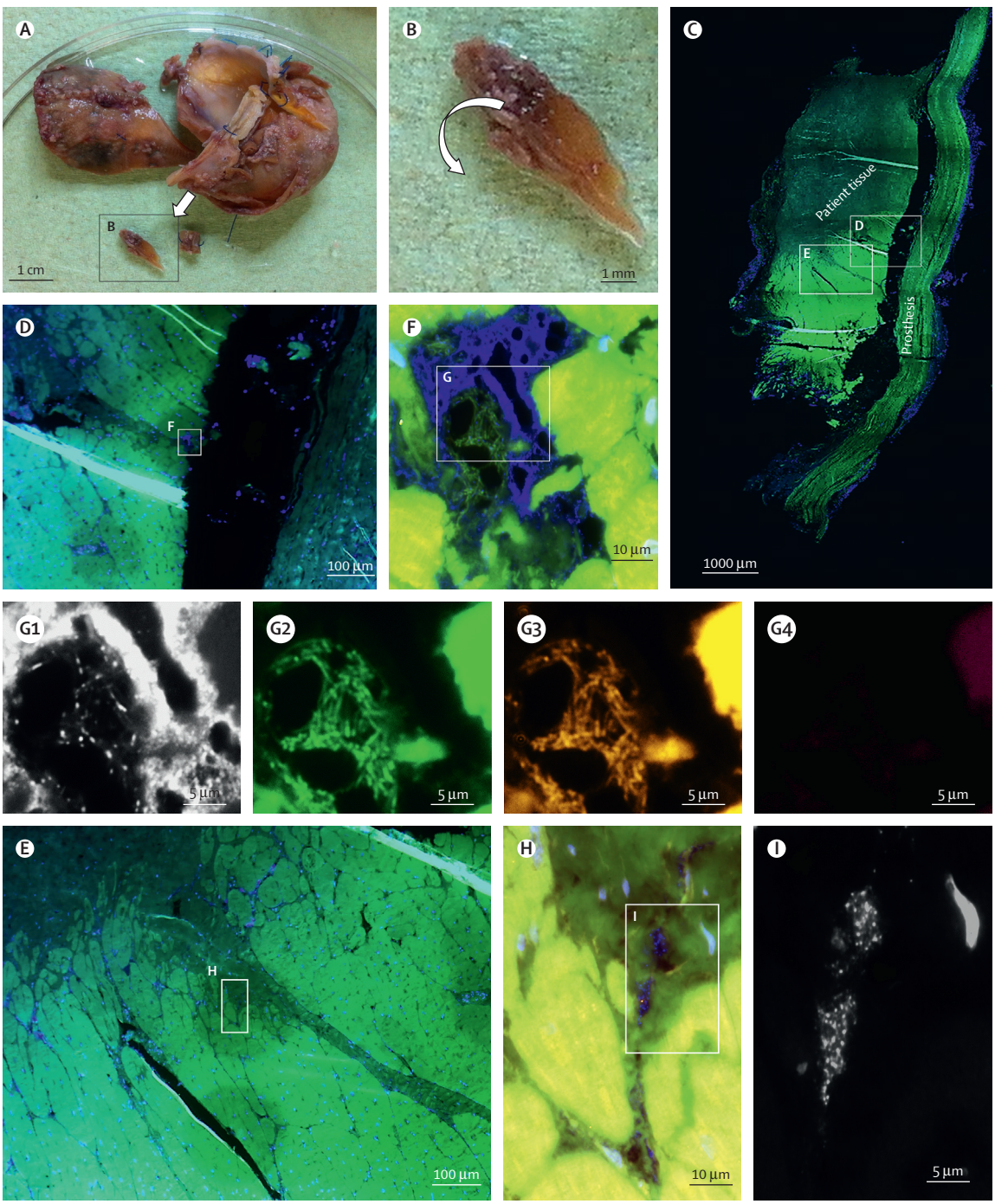

Figure 1: **FISH analysis of cardiovascular samples from patient 1**
Macroscopic images (A and B). FISH overview images (C, D, and E) show host cell nuclei stained by DAPI in blue and the autofluorescent background tissue in green. The labelled insets (F, G, H, and I) indicate regions selected for increased magnification corresponding to respective panels. Host cell nuclei and microorganisms shown in blue (DAPI), pan-bacterial PNA FISH probe shown in green (fluorescein isothiocyanate), NTM-specific PNA FISH probe shown in orange (cyanine 3), and a control PNA FISH probe detecting streptococci shown in magenta (cyanine 5; appendix p 3). (A) Macroscopic overview of the explanted heart valve prosthesis, consisting of native tissue and prosthetic material. (B) Magnification of the sample section that was embedded for FISH analysis. The sample was placed on its side in the embedding mould, so that sections contained both native tissue and prosthetic material. (C) Overview of the sample section analysed by FISH. Native tissue is shown on the left; prosthetic material is shown on the right. (D) Magnification of the interface between native tissue and prosthetic material. (F) Magnification of a region at the edge of native tissue bordering prosthetic material. The image features an opening in the tissue containing nucleic acids (blue) and rod-shaped microorganisms (blue and green). (G) Separate filter sets showing FISH-positive NTM: nucleic acid stain DAPI stained rods (white; G1); pan-bacterial PNA FISH probe showing bacteria and autofluorescent tissue (G2); NTM-specific

4·61–1517·84). The odds of a patient testing positive for *M chelonae* was significantly higher among those who had received BioIntegral bioprostheses than in those who had received bioprostheses from other manufacturers ($p<0·0001$).

Discussion

In this study, we report the findings of a collaborative diagnostic effort and in-depth microbiological analyses of cardiovascular samples from nine patients in Germany, suggesting an association between clinically relevant infection with *M chelonae* and bioprosthetic valves manufactured by BioIntegral Surgical. Investigations also showed contamination of unused BioIntegral protheses and no association between *M chelonae* infection and explanted prosthetic valves supplied by other manufacturers. Among 366 samples from the 269 bioprosthetic investigated valves, all six valves testing positive for *M chelonae* with FISHseq were from BioIntegral.

The findings of this current study are in line with a report from Switzerland[6] and an unpublished case from France. In the patient from Switzerland, endocarditis caused by *M chelonae* was diagnosed 21 months after implantation of a BioIntegral bioprosthesis. The pathological examination showed a necrotising xanthogranulomatous inflammation in the peri-valvular aortic area. The patient presented with macroscopic and histological signs of an infection due to atypical mycobacteria. This incidence was reported to the Swiss Agency for Therapeutic Products (Swissmedic Vk 20190123_02). In 2021, the National Reference Center for Mycobacteria in France reported one patient with endocarditis and the detection of *M chelonae* 3 months after implantation of a BioIntegral bioprosthesis (E Cambau, unpublished).

FISHseq has the unique advantage of identifying and visualising pathogens within sample tissues, ruling out contamination.[9,12,14] Additionally, this technique provides information on the number, exact localisation, and—via ribosome content—activity of the microorganisms. Therefore, FISHseq was included in the 2023 Duke–ISCVID criteria as a definitive diagnostic criterion for endocarditis.[10] Coincidentally, this technique is also optimal for the detection of mycobacteria because 2 µm tissue sections directly expose bacteria within the tissue to DNA extraction, allowing DNA extraction optimised for mycobacteria, sequencing of long reads (<500 base pairs) of the 16S rRNA gene, and microscopic visualisation of mycobacteria within the tissue, ruling out contamination during PCR processing. All cases were confirmed by use of different staining techniques and amplification assays in at least one additional independent laboratory, including the National Reference Center for Mycobacteria in Germany.

NTM are usually missed by routine diagnostic methods applied for endocarditis, given that they need specific cultivation conditions. *M chelonae* has optimum growth at 30°C and might be missed if incubated at 37°C, the usual temperature for bacterial culture. Additional factors can prevent growth in culture, including anti-infective treatment of patients, biofilm formation, previous treatment of bioprostheses with glutaraldehyde–formaldehyde, and adaption to the transport medium containing 2% benzyl alcohol.[4–6,15–18] Therefore, negative cultures do not rule out the presence of mycobacteria with the capability to reactivate and cause infection.[19]

The observation of negative culture results, despite viable bacteria, has been reported for other NTM, such as *Mycobacterium leprae, Mycobacterium genavense, Mycobacterium haemophilum*, and *Mycobacterium tilburgii*, which all show hardly any or no growth in culture. Furthermore, culture failure is a well known occurrence in endocarditis. Species that are usually easy to cultivate, such as streptococci, staphylococci, or enterococci, might not grow in culture due to previous antimicrobial therapy, biofilm formation, or both.[9] In the patient with reported *M chelonae* infection from Switzerland, NTM was only detected by metagenomics after culture and amplification methods had failed.[6] Therefore, it can be assumed that routine culture methods will, in most cases, miss these bacteria.

The essential question is whether the mycobacteria in prosthetic material were viable at the time of implantation and which, if any, consequences this might have for the patient. Of note, all patients received an explantation of the bioprostheses because of dysfunction, suture dehiscence, leakage, or suspected infection. We investigated samples using FISHseq and additional histological examination. Microscopic evaluation showed that the mycobacteria were associated with tissue damage and granulomas in native tissue; were morphologically intact; contained enough ribosomes to elicit a FISH signal even 4 years after implantation in patient 1; and were found not only in prosthetic material, but also in numerous microcolonies (ie, small groups of bacteria) between cardiac muscle cells in native tissue, indicating invasive growth by viable NTM. Taken together, these observations can only be explained by NTM viability with active growth in the host tissue, triggering a reaction of the host immune system. The dehiscence described for some BioIntegral bioprostheses could be due to the presence of NTM.[4–6,15–17]

By contrast, we did not find any mycobacteria in 35 patients with BioIntegral prostheses (60 samples) during routine FISHseq. This finding can be explained by either particular valve lots without NTM contamination or host immune reactions suppressing the infection, given that

PNA FISH probe showing identical bacteria (G3); and streptococci-specific PNA FISH probe showing no bacteria (G4). (E) Magnification of a region indicated in C within native muscular tissue. (H) Magnification showing rod-shaped bacteria in native tissue. (I) Magnification of rod-shaped bacteria stained by DAPI nucleic acid stain (white). Bacteria show DAPI signal but no FISH signals, indicating low ribosome content (single channels not shown). DAPI=4′,6-diamidino-2-phenylindole. FISH=fluorescence in-situ hybridisation. NTM=non-tuberculous mycobacteria. PNA=peptide nucleic acid.

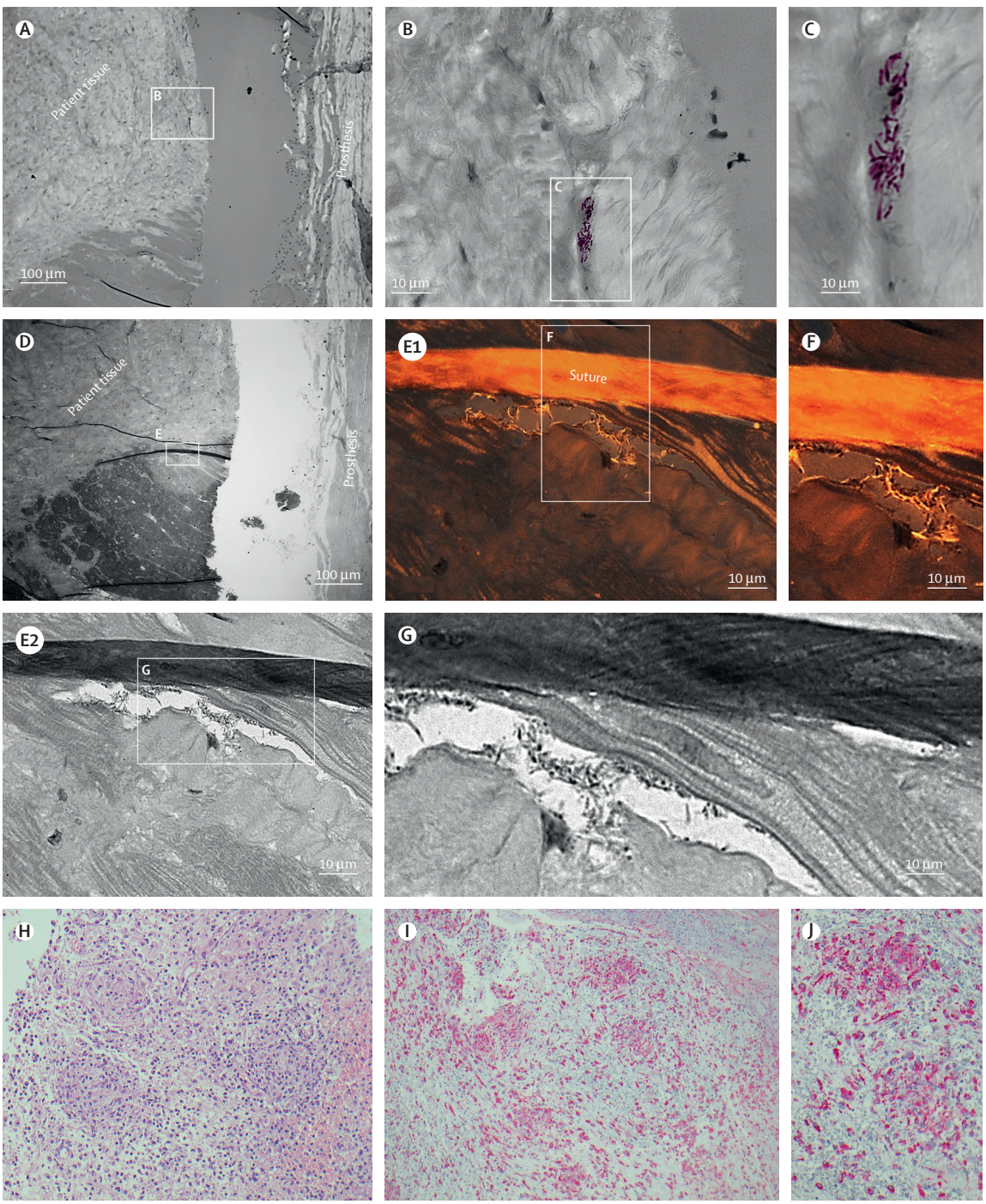

Figure 2: **Kinyoun and auramine stain of acid-fast bacilli and histological examination of cardiovascular samples from patient 1**
Labelled insets indicate regions selected for increased magnification corresponding to respective panels. (A) Kinyoun stain for the detection of acid-fast bacilli in native tissue and prosthetic material. (B) Magnification of native tissue featuring mycobacteria. Structures with high grey-scale values are false-coloured in dark red. (C) Increased magnification of mycobacteria in native tissue. (D) Auramine stain for the detection of acid-fast bacilli in native tissue and prosthetic material. (E) Mycobacteria near a suture close to cardiomyocytes; torn tissue is associated with the presence of mycobacteria. (E1 and F) Brightfield and fluorescence overlay with auramine stain showing fluorescent mycobacteria. (E2 and G) Brightfield with auramine stains showing mycobacteria in a rupture of cardiomyocytes. (H) Haematoxylin and eosin staining. (I and J) Anti-CD68 immunostaining of native tissue showing granulation tissue with formation of epithelioid granulomas on the host side.

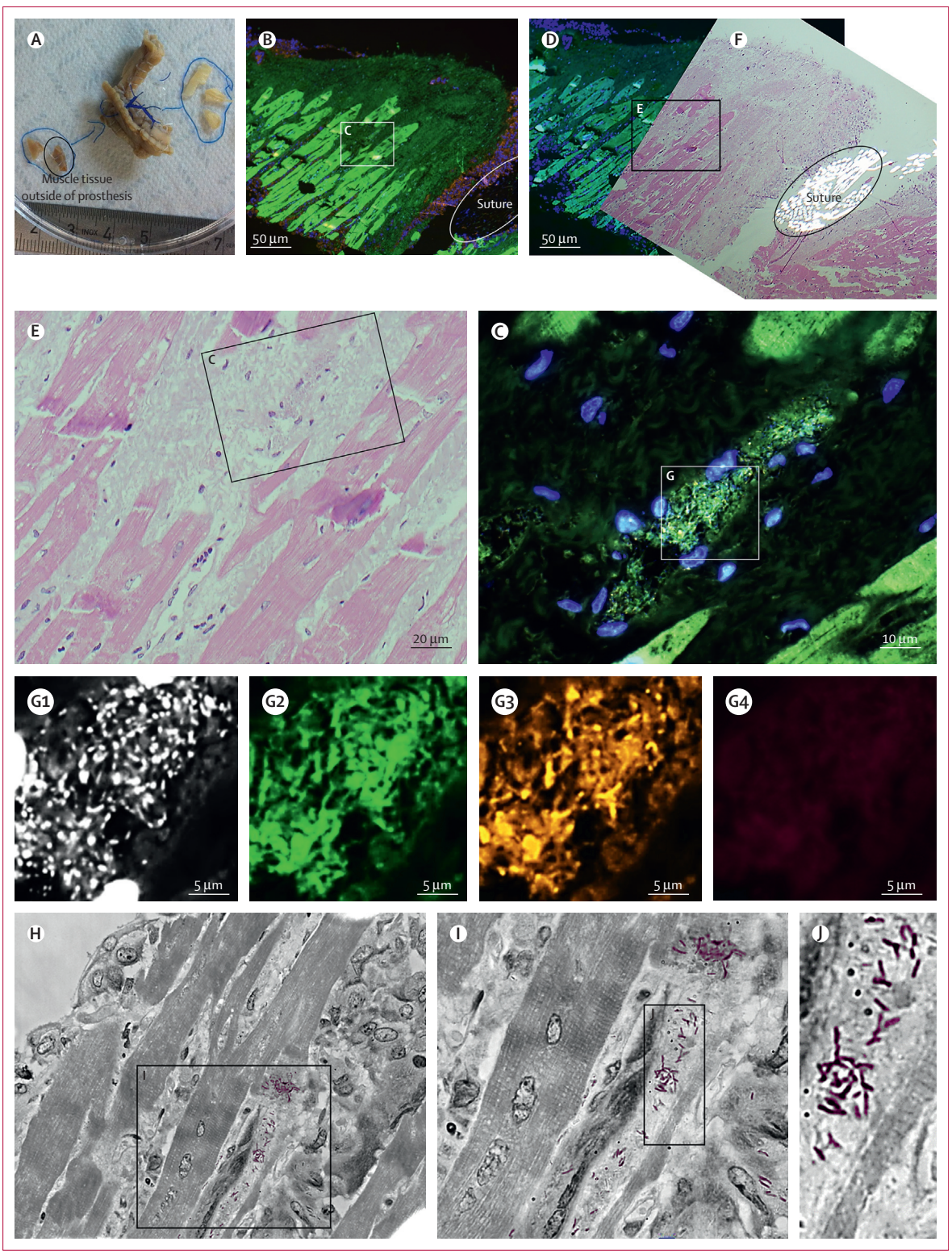

Figure 3: **NTM in heart muscle tissue of patient 3**

Host cell nuclei stained by DAPI shown in blue and the autofluorescent background tissue shown in green. (A) Overview of the explanted heart valve prosthesis, consisting of native tissue and prosthetic material. The sample sections of valve leaflets on the right side and of native tissue on the left side that were separately embedded for FISH analysis are indicated by blue circles. The sample specimen from native tissue adjacent to the prosthesis is indicated by the black circle. (B) Overview of FISH analysis showing native heart muscle tissue (material from black circle in A). FISH analysis (D) in combination with H&E stain overlaid at the corresponding position (F) of B. Cross-section of

	Native cardiovascular tissue*	Mechanical cardiovascular prostheses	Biological cardiovascular prostheses†		Total
			Other manufacturers	BioIntegral Surgical	
FISHseq-negative	232/174	71/41	73/49	30/13	406/277
DAPI-positive only	147/125	52/33	78/52	9/4	286/214
FISHseq-positive (other than NTM)	314/283	55/46	144/127	23‡/19§	536‡/475§
FISHseq-positive for *Mycobacterium chelonae*	0	0	0	11‡/6§	11‡/6§
Total	693/582	178/120	295/228	71‡/41§	1237‡/971§

Data are number of samples/number of cases. A case was defined as a single specimen of native cardiovascular tissue or prosthetic material. From each specimen or prosthesis, one or more samples from different locations were taken for FISHseq analysis. FISHseq=fluorescence in-situ hybridisation combined with broad-range 16S rRNA gene PCR and sequencing. DAPI=4′,6-diamidino-2-phenylindole. NTM=non-tuberculous mycobacteria. *Mainly heart valve tissue. †366 samples from 269 cases. ‡Including two samples positive for both *M chelonae* and *Staphylococcus aureus*. §Including one case positive for both *M chelonae* and *S aureus*.

Table 2: **Results of cardiovascular specimens investigated by FISHseq**

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M chelonae is a pathogen of low virulence. In patients 2 and 4, other species were detected by culture only, rendering a different focus of infection more likely. In patient 5, *S aureus* was the leading pathogen both in culture and FISHseq, and the clinical relevance of *M chelonae* remains unclear. However, it is worth considering whether already explanted BioIntegral prostheses should be re-examined by use of FISHseq to rule out active NTM infection in alive patients. Furthermore, it remains to be seen if host immune reactions to the presence of viable and non-viable NTM lead to inflammatory processes, resulting in an increased risk of device dysfunction or secondary infection.

Notably, among 269 bioprosthetic valves investigated, all six valves testing positive for *M chelonae* by FISHseq were from BioIntegral. Additionally, among a total of 1237 samples, FISHseq detected either other or no bacteria in 1226 cardiovascular samples from three different heart surgery centres, including 295 samples from 228 bioprostheses supplied by other manufacturers. Of note, all these samples were processed routinely with FISHseq, and none were specifically investigated with mycobacteria-specific FISH or PCR originally. Therefore, we cannot exclude that we might have missed cases. However, all samples were processed using the same methods. We performed statistical analyses to support our microbiological and clinical findings and found a significant correlation between BioIntegral bioprostheses and detection of *M chelonae*. Thus, contamination during surgery in different centres or PCR contamination in different laboratories seems highly unlikely.

Regarding the standpoint of BioIntegral Surgical, the company published regular updates regarding the ongoing *M chelonae* investigation on their website, starting with initial findings from March, 2022, and the field safety notice published on April 13, 2022.[20] In the most recent statement published on June 1, 2023, in the form of frequently asked questions, BioIntegral Surgical stated: "It's clear that no product had viable microorganisms, of any kind. The sterility processes used at BioIntegral Surgical have been well-validated to deactivate mycobacteria and more chemically-resistant organisms, and years of test results support their effectiveness." Additionally, the company states, "[BioIntegral Surgical] employs an 'overkill' method for all sterilization processes, vastly increasing confidence intervals as safety. In addition, the sterility validation and verification data were reviewed, and there were no deviations. The sterility packaging validation is the most sensitive and extensive in the industry. No other heart valve company uses such a robust system to ensure all sterilization systems are functioning properly."[21]

M chelonae has previously been suspected of being a contaminant in bioprosthetic cardiovascular devices.[7,8,22] However, the origin of the mycobacteria on prosthetic valves is still under debate; porcine or bovine valves might be harvested from colonised donor animals, or contamination might occur at the harvest or manufacturing site during production.[4]

Most bioprosthetic heart valves are treated with glutaraldehyde for decontamination. Unlike other manufactures, BioIntegral devices are treated with a heparin rinsing technique (No-React; BioIntegral Surgical, Mississauga, ON, Canada) after crosslinking with glutaraldehyde, and are packaged in an alcohol solution. BioIntegral Surgical, as well as its predecessor Shelhigh, claim their devices have a higher compatibility with host cells due to the No-React treatment than do products supplied by other manufacturers.[23] There are conflicting data on the sterility and safety of bioprostheses treated with No-React, with both favourable reports[24–26] and concerns, including warnings from the US Food and Drug Administration in 2000, 2005, and 2008 against Shelhigh.[27–32] However, to date, an infectious agent has not been confirmed.

The devices included in this study are from different lot numbers. Hence, with the current information available, it is not possible to establish whether the contamination could be attributed to specific lot numbers or production periods.

the suture in B indicated by a white ellipse and of that in F by a black ellipse. (E) Magnification of the H&E stain for detecting mycobacteria in native tissue showing disruption to the regular cardiomyocyte alignment where mycobacteria are present. (C) Magnification of the field of view in B showing the FISH analysis, corresponding to the position of the inset C in E showing the H&E stain. Host cell nuclei and microorganisms are visible in blue (DAPI), pan-bacterial PNA FISH probe and autofluorescent tissue background shown in green (fluorescein isothiocyanate), NTM-specific PNA FISH probe shown in orange (cyanine 3), and a streptococci-specific PNA FISH probe in magenta (cyanine 5; appendix p 3). (G) Separate filter sets showing FISH-positive NTM: nucleic acid stain DAPI stained rods (white; G1); pan-bacterial PNA FISH probe showing bacteria (G2); NTM-specific PNA FISH probe showing identical bacteria (G3); and streptococci-specific PNA FISH probe showing no bacteria (G4). (H) Kinyoun stain of native tissue featuring mycobacteria adjacent to disrupted heart muscle fibres. Structures with high grey-scale values are false-coloured in dark red. (I and J) Magnification of the Kinyoun stain showing clearly visible mycobacteria. DAPI=4′,6-diamidino-2-phenylindole. FISH=fluorescence in-situ hybridisation. H&E=haematoxylin and eosin. NTM=non-tuberculous mycobacteria.

Furthermore, molecular analysis of the internal transcribed spacer region found in all sequenced cases showed the same mismatch as the *M chelonae* subspecies *gwanakae* strain MOTT36W (sequence identification number CP031516.1). This finding could point to a possible clonality of *M chelonae*. However, the discriminative power of this mismatch is not known and needs further investigation.

Limitations of this study include that the diagnostic options available to detect *M chelonae* are limited to postoperative material. Therefore, clinical aspects, laboratory parameters, and diagnostic imaging (eg, [^{18}F]fluorodeoxyglucose-PET-CT) in patients need further investigation to identify criteria that justify therapeutic intervention.

The culture results were negative, which makes a direct comparison of strains from unused prostheses and patient samples difficult. Unfortunately, no further information on the origin of *M chelonae* could be obtained because the manufacturer did not allow an investigation of the production process.

The German Federal Institute for Drugs and Medical Devices and BioIntegral Surgical were informed about the findings, leading to a field safety notice to be published in April, 2022.[33] This action was followed by a general notification by the German Society for Thoracic and Cardiovascular Surgery and other European societies and competent authorities, warning public health authorities against use of these products. Discussions with international experts and competent authorities are ongoing regarding more cases, as well as favourable diagnostic and treatment practices.

Preventive reoperation of asymptomatic patients with a functioning BioIntegral bioprosthesis and without signs of structural valve failure or graft infection cannot be advised, given that there is currently no clear preoperative evidence of systematic graft-to-host infection. Pre-emptive antimycobacterial treatment is a challenging decision because it consists of a combination of different antibiotic compounds with serious side-effects and long treatment duration. Furthermore, treatment is based on empirical experience because susceptibility testing is not feasible.

Our findings should alert clinicians about the potential risk in patients with BioIntegral prostheses and either suspected infection or dysfunction, such as valve degradation, dehiscence, obscure mass associated with the graft, or a combination of all three. This patient group should be closely followed up with extended diagnostics—eg, mycobacterial blood culture, medical imaging by PET-CT, and, if the tissue is available, mycobacterial diagnostics (including PCR and culture), FISHseq, and histological examination (appendix pp 23–25). In all patients, clinical decision making requires careful balancing of potential risks and benefits by an experienced team of endocarditis specialists. International cooperation to collect all available information and cases will be essential to further establish the effects of bioprosthesis contamination with *M chelonae*. Therefore, an international prospective register (MYBACH; registered at German Clinical Trials Register) has been established at Charité–Universitätsmedizin Berlin (number DRKS00034062).

In conclusion, molecular investigation involving FISHseq showed the contamination of BioIntegral bioprostheses with *M chelonae* in a case series of nine patients from Germany and in 12 unused prostheses. Clinicians should be aware of the potential association between presence of *M chelonae* in bioprosthetic valves and the risk of implant dysfunction. Standardisation bodies and competent authorities should review the current standards and regulatory requirements on medical devices that use tissues of animal origin to reflect these findings.

Contributors

JK, IF, KS, SW, LK, DvS, MK, and AM accessed and verified all the data in this study. JK, IF, SW, LK, and AM conceptualised the study. JK, IF, LK, DvS, DH, MK, and AM curated the laboratory data. CL curated the pathology data. SW, KS, MSS, FP, F-RK, VD, EC, ST-S, BHa, AF, BP, WE, VF, and PG curated the clinical data. JZ, BHä, EPCT, SH, AvL, and TE curated the regulatory and public health data. JK and AM acquired funding for the study. JK, IF, KS, LK, DvS, DH, MK, and AM were responsible for the laboratory analysis. CL was responsible for the pathology methodology. SW, MSS, FP, F-RK, VD, EC, ST-S, BHa, AF, BP, WE, VF, and PG were responsible for patient care. AM was responsible for project administration. JK and AM wrote the original draft. All authors contributed to the investigation, validated the study, and reviewed and edited the manuscript. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Declaration of interests

JK and AM founded and run MoKi Analytics GmbH. AM founded and runs Moter Diagnostics. These two diagnostics laboratories investigated the samples with FISHseq originally and, after finding NTM, initiated and handed over this collaborative research to all other partners.

Data sharing

Individual de-identified patient data are available in the appendix (pp 7–10). Additional data cannot be shared due to patient confidentiality and consent.

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