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Application of poly-L-lactide screws in flat foot surgery: histological and radiological aspects of bio-absorption of degradable devices

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Summary. The flat foot in childhood is a condition frequently observed in orthopedic practice but it is still debated when and in which patients surgical corrective treatment is appropriate; recently, the application of poly-L-lactic-acid (PLLA) screws was proposed. The present study investigates a group of 33 patients treated with PLLA expansion endorthesis in order to evaluate the deformity correction. Clinical and radiological outcomes in patients were correlated with: a) morphological characterization of screws both before and after being removed from patients, when necessary; b) histological and bio-molecular evaluation of degradation processes of the implants, focusing attention on the correlation between the cellular cohort involved in inflammatory reaction and the bio-absorption degree of PLLA screws. Deformity correction was mostly achieved, with minimal need of screw removal; the results obtained clearly show the occurrence of chronic rather than acute inflammation in removed screw specimens.

At the histological level, after biomaterial implantation, the sequence of events occurring in the surrounding tissues ultimately ends in the formation of foreign body giant cells (FBGCs) at the tissue/material interface; but the mechanisms which influence the fate of screw implants, i.e. the resolution of acute inflammation rather than the progression towards chronic inflammation, are of crucial importance for biodegradable materials like "polylactic acid". In fact, the FBGC response ensures a long-term mechanism which eliminates the foreign material from the body, but at the same time the implications of prolonged FBGC responses, which generate negative side effects, could significantly impede the healing progress.

Key words: PLLA screws, Flat foot, Bio-absorption, Histological analysis

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Introduction

The flat foot in childhood is a condition frequently observed in common orthopedic practice. It is characterized clinically by a lower medial arch, which is stressed by monopodalic weight bearing. From a biomechanical point of view, the foot is comparable to a propeller with two blades which twist and untwist harmoniously through every step (Paparella Treccia, 1977) and the pivotal morphofunctional device of this sequence of movements is the subtalar joint. This joint can make two triplanar movements: supination and pronation. In the flat footed child, the pronation component prevails over the supination one and in the most severe cases the pronation component is the only one performed. Therefore, another definition of flat foot could be a foot in a prevailing or persisting pronation state (Giannini et al., 2001). At the present stage it is still debated when and in which patients surgical corrective treatment with arthroereisis of the subtalar joint is appropriate. Although many authors (Giannini et al., 1991; Gutiérrez and Herrera, 2005; Needleman, 2005) have pointed out that restoring the relation between the talus and the heel is necessary to prevent the secondary problems related to excessive pronation, which can

eventually become evident in adulthood (especially when a serious clinical pronation coexists with an alteration of the principal weight bearing radiological axes), the best solution in the arthroereisis of the subtalar joint is the correction of the flexible flat foot in children. This treatment, based on the use of metallic screws implanted in the external part of the sinus tarsi, was described for the first time by Burutaran (1979).

Poly L-lactic acid (PLLA) is considered a suitable screw component because it is a biodegradable and bioadsorbable semicristalline homopolymer of PLLA, with different stereochemistry features. The degrading rate of PLLA is very slow, with reported values of degradation time needed of 2 to 6 years (Maurus and Kaeding, 2004), strongly dependant on the degree of polymer crystallinity, as well as the properties of the polymeric formulation, i.e. structure and porosity of the device. Good tensile strength, low extension and high modulus configure PLLA as the ideal biomaterial for load bearing applications. The *in vivo* degradation of PLLA occurrs not only by chemical mechanisms, but also by enzymatic pathways (Williams, 1981). Under the mere chemical aspect, this process is controlled by main parameters: the constant rate, the site of implantation and its vascularization and the amount of absorbed water. The biodegradation of a polymer is a very complex process, at the end of which acidic products accumulate centrally into the implant, thus lowering the pH in the bulk of the material; this phenomenon is accompanied with a burst of acidic products released in the surrounding area and a dramatic loss in mechanical properties that may be responsible for the observation of delayed inflammatory response and mechanical failure respectively (Li et al., 1990; Athanasiou et al., 1998; Böstman and Pihlajamaki, 2000a,b). Host reactions following implantation of biomaterials include injury, blood-material interactions, provisional matrix formation, acute inflammation, chronic inflammation, granulation tissue development, foreign body reaction and fibrosis or fibrous capsule development (Anderson, 2000, 2001). In the early phases after implantation, blood/material interactions occur with both protein absorption to the biomaterial surface and development of a transient provisional matrix that forms on/around the biomaterial. The provisional matrix provides structural, biochemical and cellular components for the process of wound healing and foreign body reaction. The presence of mitogens, chemotactic molecules, cytokines, growth factors and other bioactive agents within the provisional matrix determines the activation or inhibition of substances capable of modulating macrophage activity, along with the proliferation and activation of other cell populations in the inflammatory response and wound healing process (Gretzer et al., 2006; Luttikhuizen et al., 2006).

Following the initial blood/biomaterial interaction, acute and chronic inflammations occur in a sequential manner. The degree of these responses is controlled by the extent of injury during the implantation procedure, as well as the type of tissue/organ into which the device is implanted. The presence of neutrophils characterizes the acute inflammatory response. Mast cell degranulation and the subsequent release of histamine and fibrinogen absorption are known to mediate acute inflammatory responses to implanted biomaterials (Tang et al., 1998; Zdolsek et al., 2007). Biomaterial-mediated inflammatory responses may be modulated by histamine-mediated phagocyte recruitment and phagocyte adhesion to implant surfaces facilitated by adsorbed host fibrinogen (Wilson et al., 2005). The acute inflammatory process usually resolves quickly, depending on the extent of injury at the implant site. Following acute inflammation, chronic inflammation is identified by the presence of monocytes and lymphocytes at the implant site. This chronic inflammatory response to biomaterials is usually of short duration and is confined to the implant site. Chronic inflammation justifies the prolonged presence of foreign body reaction where monocytes, macrophages and foreign body giant cells (FBGCs) are present at the biomaterial interface. Moreover, granulation tissue development is identifiable by fibroblast infiltration and a newly-vascularized area in the new healing tissue (Chen et al., 2007). The mechanisms which influence the fate of the local response to biomaterial implantation, i.e. the resolution of acute inflammation rather than progression towards chronic inflammation, are of critical importance in understanding the sequence of events following the introduction of biodegradable materials in the body. Considering this, the main aim of the present study is to investigate a group of patients treated with PLLA expansion endorthesis in order to compare the histological aspects and the morphological SEM (Scanning Electron Microscopy) observations of the bio-absorption process of PLLA devices in relation to the clinical outcome, focusing attention particularly on the correlation between the cellular cohort involved in inflammatory reaction and the bio-absorption degree of PLLA screws.

Materials and methods

Surgical technique

During this study 33 patients (20 males, 13 females) were surgically treated and reviewed for a total of 59 feet: in 26 patients the surgical treatment was bilateral, in 7 patients monolateral. The follow-up, as far as the clinical evaluation is concerned, ranged from 35 to 104 months. The follow-up was variable because each patient was reviewed from the clinical point of view for the first time after three years, and then at different times depending on the individual response to the discomfort due to incomplete screw reabsorption. Moreover, it has to be considered that the examined population mainly includes children, whose motor activity and individual susceptibility to pain are very different. All patients were surgically treated for child flexible idiopathic flat foot at

the Orthopedic Clinic of the Policlinico of Modena between January 2002 and May 2007. For correction of the flat foot, we used the PLLA bioabsorbable expansion endorthesis (PLLA-Purasorb PL65 screw, Howmedica Stryker, Allendale, NJ; inherent viscosity -IV- between 5.8 and 7.2 dl/g, medium molecular weight -mMW- 1500000 g/mol). Two screw diameters were used: 8 and 10 millimeters (the screw diameter choice was based on both the severity of the pathological condition and the degree of correction to be obtained). The mean age at surgery was 11.8 years (min. 8.2, max. 16.1) and the mean follow-up period at the clinical and radiological examination was 52 months (min. 35, max.104). The 8 mm diameter screw was used in 31 cases, the 10mm diameter screw was implanted in 28 cases.

All the patients enrolled in this study gave informed written consent to the study protocol, which was approved by the local Ethical Committee.

Clinical evaluation of flat foot correction

The clinical evaluation was conducted using an evaluation score that takes into account the main clinical, biomechanical and functional parameters of the foot. The score ranges from 0 to 100 points, considering the following parameters: 1) Pain during jogging and/or normal walking (10 points); 2) Resumption of sport practice (10 points); 3) ROM (Range Of Motion) of the subtalar joint (15 points); 4) Functionality of the forefoot position/first metatarsal position (15 points); 5) Position of the heel with bearing weight (15 points); 6) "Ballet" test (with podoscope) (15 points); 7) Functionality of the Achilles tendon (10 points); 8) Sensitivity to ground texture (10 points). The radiological evaluation was conducted by mean of two radiological orthostatic projections: lateral and dorso-plantar. The four principal angles were measured in order to evaluate whether or not the correction had occurred. In the lateral projection 3 angles were computed: 1) Costa-Bertani angle (normal value 120-125 degrees); 2) Meary angle (n.v. greater than 170 degrees); 3) Calcaneal pitch angle (n.v. 15-20 degrees). In the dorsoplantar projection the Kite angle was measured (n.v. 20-25 degrees). Based on the radiological results, the patients were divided into 4 groups: very good (with four correct angles), good (when 3 out of four angles were in the normal range), fair (with two normal angles), poor (with only one normal angle).

Evaluation of screw bio-absorption

MRI analysis

The evaluation of screw bio-absorption was conducted through Magnetic Resonance Imaging (MRI), comparing the images obtained by coronal, sagittal (anteroposterior) and transverse projections. Five stages were assigned, based on the percentage of bio-absorption: Stage 1 (bio-absorption less than 25% of the screw); Stage 2 (bio-absorption between 25% and 50%); Stage 3 (bio-absorption between 50% and 75%); Stage 4 (bio-absorption greater than 75%); Stage 5 (complete bio-absorption).

Western Blot analysis (bio-molecular evaluation)

The total cell lysates obtained from soft tissue samples, taken during screw removal operations, were extracted with hypotonic buffer. Lysates were then cleared by centrifugation for 15 min in a refrigerated centrifuge and immediately boiled in SDS sample buffer. From each sample 40 μg of extracted proteins were electrophoresed on SDS-PAGE and transferred to nitrocellulose membranes. The protocols of the Western blot were performed as described classically by Sambrook et al. (1989). The membranes were incubated with the following antibodies, diluted 1:1000 overnight at 4°C under agitation: anti-mouse CD68 (SIGMA) as marker of macrophages/FBGCs, anti-rabbit CD3 (Cell SIGMA) as marker of T-lymphocytes, anti-rabbit myeloperoxidase (SIGMA) as marker of neutrophyls. After washing, the membranes were incubated with secondary HPR-conjugated goat anti-rabbit IgG antibody (1:10000) or HPR-conjugated sheep anti-mouse IgG antibody (1:3000) for 30 min at room temperature. Immunoreactive proteins were detected with ECL (Amersham). Furthermore, the membranes were stripped and incubated with anti-mouse ß-tubulin (SIGMA) in order to control and correct for loading error.

SEM evaluation

Fragments of screws removed at stages 3-4 of bio-absorption were analyzed. The microscopic aspects of the screw fragments extracted from the patients were compared with the original unused screws by scanning electron microscopy (SEM, XL-40 Philips, Eindhoven, The Netherlands); the outer surface (i.e. that in contact with the tissue) and the inner surface (i.e. that located deeper with respect to the biological environment) were analyzed. The samples were dried and coated with carbon in a Carbon Coating Device (Balzers CED-010).

Histological analysis

Surgical specimens of soft tissue (from patients with screw removal) adjacent to the fragments of screws were observed by light microscopy (Zeiss Axiophot, Jena, Germany). Light microscopy was performed on fixed and paraffin-embedded specimens at a 20 and 40-fold magnification on three different Hematoxilin- Eosin (H&E)-stained slices of the same block, and micrographs were recorded from 10 distinct areas of the same slice. In the histological evaluation, the following considerations were taken into account: a) the density of neutrophils reveals acute inflammation; b) lymphocytes are consistent with cell immune response; c)

macrophages plus foreign body giant cells indicate the occurrence of chronic inflammation.

Semiquantitative evaluations were performed on H&E stained slices examined by light microscopy and recorded by an Image Analysis System (NIS software, Nikon, Tokyo, Japan). For each patient, ten microscopic fields, including screw remnants, were randomly selected for comparative evaluations. In each microscopic field, the following areas were measured: a) total area, b) area occupied by macrophages/FBGCs, c) area occupied by screw remnants. The percentage of the following ratios were calculated: a) area occupied by macrophages/FBGCs on total area (% M-FBGC/TA), b) area occupied by screw remnants on total area (% SR/TA). The values were expressed as mean ± standard error of mean (SE).

Statistical analysis

Statistical differences between % M-FBGC/TA and % SR/TA were analysed by Kruskal-Wallis non parametric test, followed by the Dunn test for multiple comparisons. Differences were considered significant for $p<0.05$.

The correlation between stage and time of bio-absorption was analysed using a linear regression curve; correlation was considered significant for $p<0.05$ and very significant for $p<0.01$.

Results

Correction of flat foot

The cases studied are reported in Table 1. They consist of 33 patients, identified with progressive numbers; for each of them, the following characteristics were recorded: the diameter of the screw implanted, the number of months of follow-up, the stage of the MRI, the clinical and radiological final results, and the removal of screws (if performed). The deformity correction was mostly obtained, since 29 patients (88%) showed a very satisfactory result from the clinical point of view; 3 patients (9%) showed fair clinical results; in one case (3%) the deformity was only partially corrected, with a poor score. The radiological evaluation of the correction was in accordance with the clinical

Table 1. Summary data of the 33 cases studied.

Pt.	Ø SCREW	FOLLOW UP	Stage1-5 (MRI)	CLINICAL SCORE	RADIOLOGICAL EVALUATION	NOTES
1	8 DX-SX	44	dx 4, sx 4	98	VERY GOOD	
2	10 DX-SX	48	dx 3, sx 3	100	VERY GOOD	
3	10 DX-SX	49	dx 2, sx 3	100	VERY GOOD	
4	8 DX-SX	62	dx 4, sx 4	93	GOOD	
5	10 SX	40	sx 3	100	FAIR	
6	10 DX-SX	46	dx 4, sx 4	65	FAIR	Removal of the screws
7	8 DX-SX	35	dx 3, sx 4	95	VERY GOOD	Removal of the screws
8	8 DX	35	dx 4	90	VERY GOOD	
9	8 DX-SX	67	dx 4, sx 4	95	VERY GOOD	
10	8 DX-SX	46	dx 4, sx 3	95	VERY GOOD	
11	10 DX 8 SX	60	dx 4, sx 4	100	VERY GOOD	Removal of the screws
12	10 DX-SX	83	dx 5, sx 5	95	GOOD	
13	8 SX	36	sx 3	100	VERY GOOD	
14	10 DX-SX	44	dx 3, sx 3	85	VERY GOOD	Removal of the screws
15	8 DX 10 SX	56	dx 4, sx 4	93	VERY GOOD	
16	10 DX-SX	50	dx 4, sx 4	75	VERY GOOD	
17	8 DX-SX	104	dx 4, sx 4	100	VERY GOOD	
18	8 DX 10 SX	35	dx 3, sx 3	95	VERY GOOD	Removal of the screws
19	8 DX-SX	74	dx 4, sx 4	95	VERY GOOD	
20	10 DX	43	dx 2	98	VERY GOOD	Removal of the screw
21	8 DX-SX	44	dx 4, sx 3	85	GOOD	
22	10 DX-SX	73	dx 5, sx 5	95	VERY GOOD	
23	10 DX-SX	66	dx 4, sx 4	88	GOOD	
24	10 SX	47	sx 2	80	GOOD	
25	8 SX	45	sx 3	100	VERY GOOD	
26	8 DX-SX	40	dx 3, sx 4	100	VERY GOOD	
27	10 DX-SX	44	dx 4, sx 4	93	GOOD	
28	10 DX-SX	40	dx 2, sx 2	73	GOOD	Removal of the screws
29	8 DX-SX	43	dx 3, sx 4	92	VERY GOOD	
30	10 SX	49	sx 4	85	VERY GOOD	
31	8 DX-SX	70	dx 4, sx 4	93	VERY GOOD	
32	8 DX-SX	35	dx 2, sx 3	87	VERY GOOD	Removal of the screws
33	8 DX 10 SX	63	dx 3, sx 5	72	POOR	

For each case, the following parameters are reported: progressive number indicating the patient; diameter of the implanted screws in the foot/feet surgery; follow-up months; stage of bio-absorption of the removed screws (MRI); clinical score; radiological evaluation; screw removal (if performed).

results: 30 patients (91%) showed very good radiological evidence, with restored radiological axis.

Bio-absorption of screws

The different stages of bio-absorption evaluated in our study are reported in Figure 1. The evaluation of the screw bio-absorption was conducted through magnetic resonance imaging (MRI), comparing the images obtained by coronal, sagittal and transverse projections and identified according to the 5 stages above described (1>25%; 25%<2<50%; 50%<3<75%; 4>75%; 5=100%). The instrumental evaluation of the screw bio-absorption stages with MRI showed that in 5 cases (8.5% of the

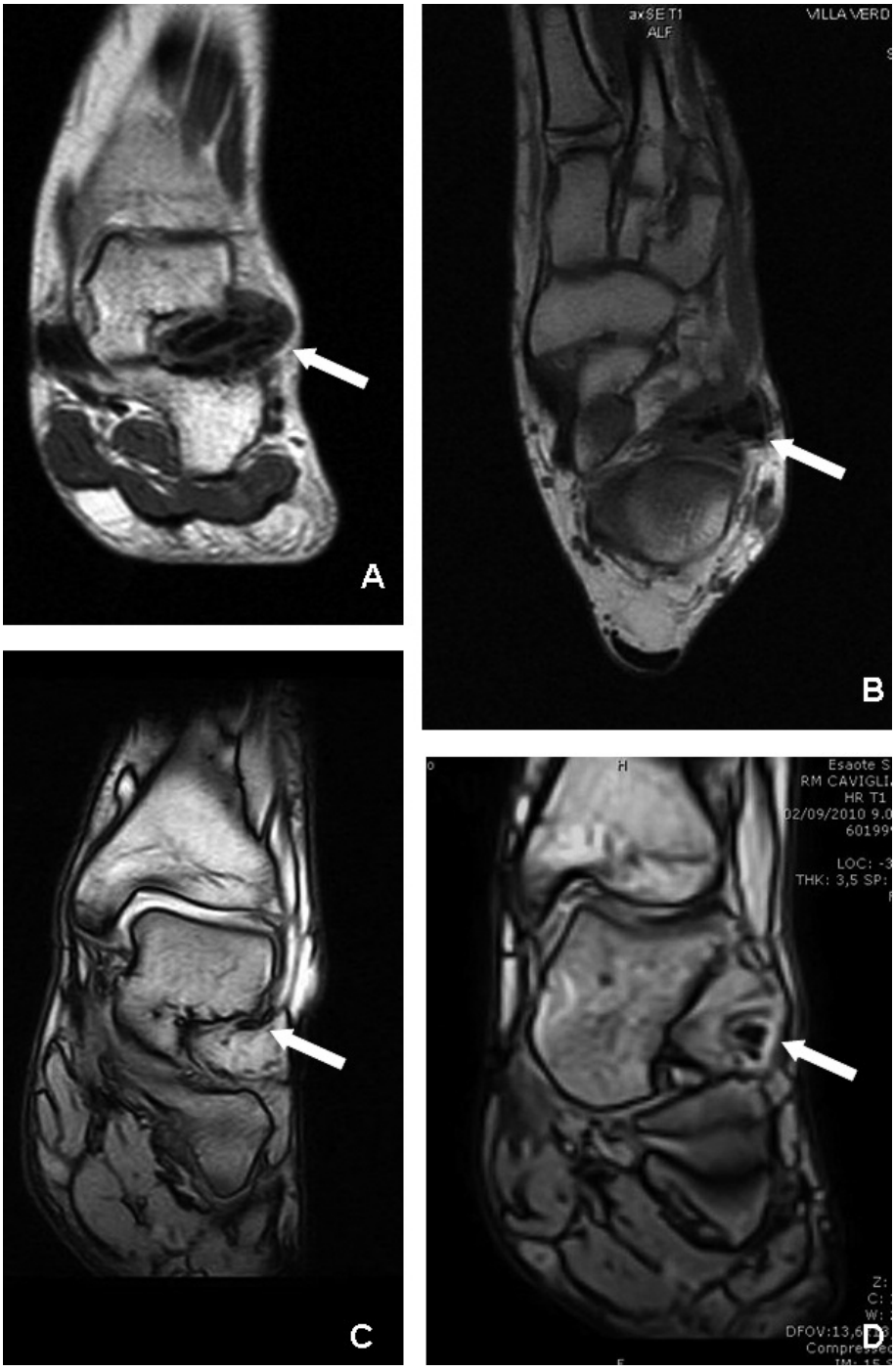


Fig. 1. Coronal sections (antero-posterior view) of 4 patients showing different bio-absorption stages of the screws: stage 2, case n° 24 **(A)**; stage 3, case n° 3 **(B)**; stage 4, case n° 1 **(C)**; stage 5, case n° 22 **(D)**. The arrows point to the implant site.

cases examined), the bio-absorption was complete (stage 5) with a mean time of 75 months; 31 cases (52.5%) were assessed at stage 4 of bio-absorption in a mean time of 57 months (with very different time values). The MRI examination highlighted that the screws at bio-absorption stages 3 and 4 were going to crack, but the remaining parts of the screws were still present, embedded in the tissue. In particular, in most cases we recognized the bio-absorption of the intermediate and the medial parts of the screw; these were the most mechanically stressed parts during the prono-supination movements of the subtalar joint. Nevertheless, after implantation the screw proved to be well seated inside the sinus tarsi, within one or two years the screw tended to lose its correct position and move externally from the sinus tarsi; this new incorrect position prevented complete bio-absorption. This condition caused discomfort for 8 patients, mainly when walking, so that it was necessary to remove the implant in 15 cases (25.4%). In 8 cases (13.6% of the total), the local adverse reaction was quite evident with a flogosis and moderately painful swelling. In all these cases, the patients were surgically re-treated and the remnants of the screws, together with the surrounding matrix/soft tissues, were removed. The comparison between the diameter of the screws (8 or 10 mm) and both the stage and the mean time of bio-absorption showed that 8mm-diameter screws were bio-absorbed in a slightly higher percentage than 10 mm-diameter screws (Tables 2, 3); in particular, 8 mm-diameter and 10mm-diameter screws were observed to be in the late stages of bio-absorption in 64.5% and 57.1% of the cases, respectively, with a bio-absorption mean time of about 60 months for the former and 64 months for the latter (each average

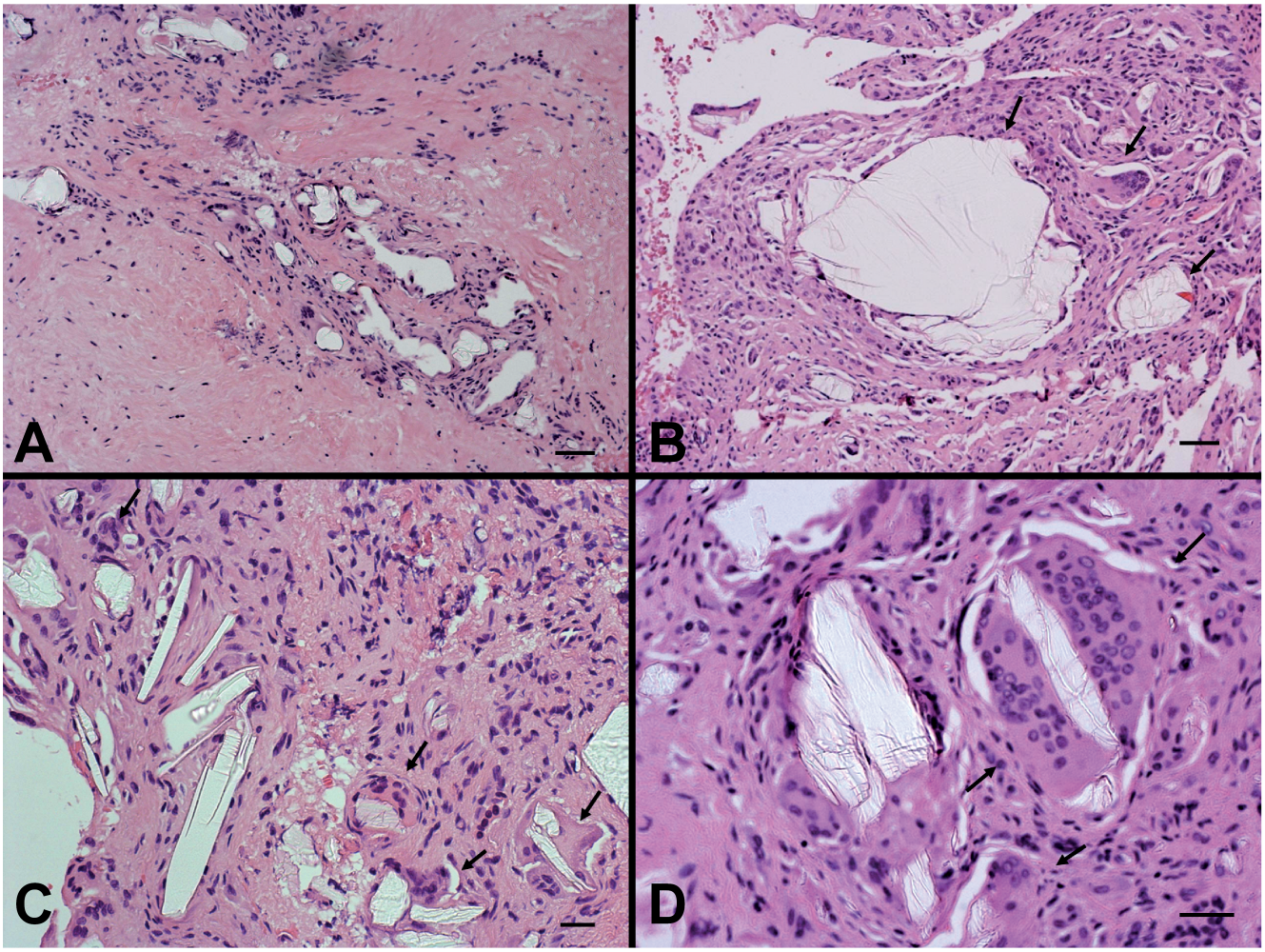


Fig. 2. Light micrographs (haematoxylin-eosin staining) of the soft tissues adjacent to screw remnants (white birifrangent material). Note the complete absence of neutrophils. **(A)**. **B.** Soft tissue showing numerous large multinucleated FBGCs (arrows) close to screw remnants. **C.** Fibrous tissue including many screw remnants surrounded by FBGCs (arrows). **D.** High magnification showing FBGCs of considerable size (arrows) surrounding screw remnants. Bar scale: A, 100 μm; B, 80 μm; C, D, 35 μm.

covering a wide range).

Analysis of the linear regression curve indicated that for both screw diameters (8 or 10 mm) a statistically significant correlation between stage and time of bio-absorption was observed, with the maximum value of significance ($p<0.01$) for 10 mm-diameter screws (Graphs 1, 2).

Concerning the 8 patients from which the screws were removed, the data shown in Table 4 clearly indicate that, independently of the screw diameter, the correlation between stage and time of bio-absorption is not significant.

Inflammatory response

The histological analysis of soft tissue specimens adjacent to implanted screws (8 or 10 mm) has been performed under light microscopy. We considered the presence and density of three different cell populations as indicative of three different conditions: a) neutrophils as a sign of acute inflammation; b) lymphocytes as a sign of cell immune response; and c) macrophages together with foreign body giant cells (FBGCs) as a sign of chronic inflammation. Briefly, the morphological evaluation showed the complete absence of neutrophils in all the specimens, indicating the absence of acute inflammation or infection (Fig. 2); the slight lymphocyte infiltration suggests that the cell-mediated immune response is significantly reduced, whereas macrophage infiltration was well detectable and numerous FBGCs were observed in dense collagenous tissue adjacent to the degrading polymer, lining the interface between tissue and polymer (Fig. 2C,D). This large amount of infiltrating FBGCs reflected the chronic inflammatory response in surrounding soft tissue. Moreover, oedema

Table 2. Data showing the bio-absorption stage of 8 mm-diameter screws (by MRI).

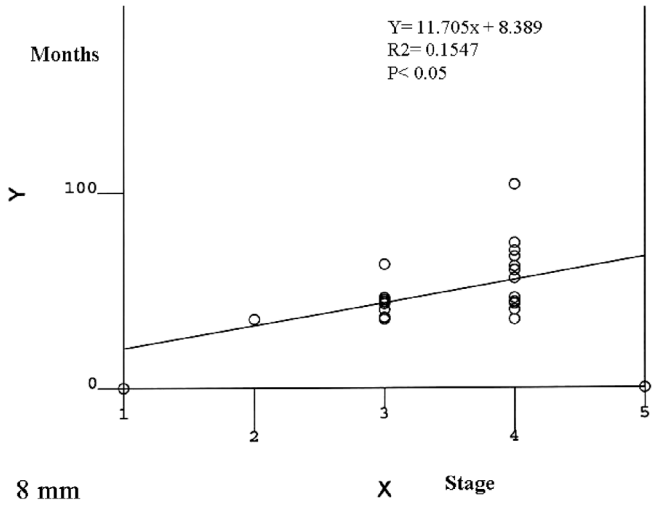
BIOABS. STAGE	N. SCREWS	%	MEAN TIME OF REABSORPTION (months) ± SE
stage 1	0	0.0	0
stage 2	1	3.2	35.0
stage 3	10	32.3	42.2±2.70.
stage 4	20	64.5	60.1±4.44
stage 5	0	0.0	0

Table 3. Data showing the bio-absorption stage of 10 mm-diameter screws (by MRI).

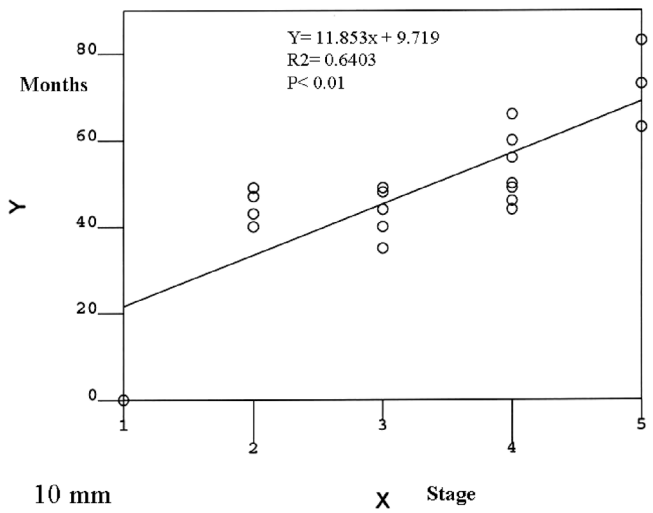
BIOABS. STAGE	N. SCREWS	%	MEAN TIME OF REABSORPTION (months) ± SE
stage 1	0	0.0	0
stage 2	5	17.9	43.8±1.83
stage 3	7	25	44.7±1.91
stage 4	11	39.2	52.4±2.5
stage 5	5	17.9	75.0±3.74

Table 4. Bio-absorption stage of removed screw (by MRI).

BIOABS. STAGE	N. SCREWS	%	MEAN TIME OF REABSORPTION (months) ± SE
stage 1	0	0	0
stage 2	4	26.67	39.5±1.66
stage 3	6	40.00	38.0±1.89
stage 4	5	33.33	49.4±4.77
stage 5	0	0	0



Graph 1. Regression curve between 8 mm-screw bio-absorption time (y axis) and MRI-stage (x axis). Squared correlation coefficient (R^2) is used as a measure of goodness-of-fit. Low p values ($p<0.05$) indicate that time of bio-absorption and MRI-stage are significantly related.



Graph 2. Regression curve between 10 mm-screw bio-absorption time (y axis) and MRI-stage (x axis). Low p values ($p<0.01$) indicate that time of bio-absorption and MRI-stage are very significantly related.

was reduced and fibrin deposit, as well as fibrinoid necrosis, did not accompany oedema, confirming relatively reduced inflammation due to biomaterial implantation. The presence of birifrangent exogen material inside FBGCs was observed in many specimens (Fig. 2B-D). The screw fragments were very variable in size, depending on the bio-absorption stage (Fig. 2A,B).

Histomorphometric data concerning the 8 patients from which the screws were removed are reported in table 5 (it is to be mentioned that histological samples of bilateral screws removed from one patient were not available at the time of data collection). With respect to the total area, the area occupied by macrophages and FBGCs ranged between 1.6% and 7.63% and the area occupied by screw remnants was detected to be very variable, ranging from 1% to 26%. The Kruskal-Wallis test followed by the Dunn test indicates that the differences between the mean percent area occupied by screw remnants (2nd column in Table 5) in those patients in which clinical evaluation was considered "fair" and "very good" are statistically significant. On the contrary, the comparison between the mean percent area occupied by macrophages/FBGCs (1st column in Table 5) does not show significant differences. No statistical significance was recorded even when all the above mentioned histomorphometric data were correlated to the stage of bio-absorption as well as the time of follow up.

To improve the identification of the cellular cohorts described by the morphological viewpoint, cell lysates of the same soft tissues obtained during screw removals were analysed by Western blotting in order to reveal the marker profile of the inflammatory cells: the anti-CD68 protein was used to identify macrophages; the anti-CD3 protein and anti-myeloperoxidase protein were used to assess the presence of lymphocytes and neutrophils, respectively. The results clearly showed a single intense band at the expected molecular weight (37 kDa) for CD68; on the contrary, very low bands were detected for CD3 (20 kDa) and myeloperoxidase (150 kDa), as shown in Figure 3. No considerable differences were recorded when the marker profiles were compared in relation to the screw diameter.

Table 5. Data showing the mean percentage area occupied by macrophages/FBGC and by screw remnants in removed samples, the radiological evaluation, the follow-up, the MRI-stage.

Sample	% M-FBGC /TA ± SE	% SR/TA ± SE (*)	Radiological evaluation	Follow-up months	Stage MRI
1	4.40±0.92	11.35±4.49	fair	46	4
2	3.92±0.90	26.01±9.06	fair	46	4
3	6.59±1.53	1.05±0.46	very good	35	3
4	7.63±1.92	11.76±7.86	very good	35	3
5	4.43±0.96	4.60±1.69	very good	35	3
6	4.44±0.89	2.16±1.18	very good	35	4
7	2.02±0.53	1.87±0.82	very good	60	4
8	3.03±0.85	3.56±1.03	very good	60	4
9	1.60±0.53	1.42±0.34	very good	44	3
10	2.20±1.83	2.03±1.21	very good	44	3
11	4.12±1.62	4.55±0.95	very good	43	2
12	3.78±0.85	4.02±1.91	very good	35	2
13	3.55±1.10	2.91±1.37	very good	35	3

(*) % area of screw residues in fair samples versus very good samples was statistically significant ($p < 0.05$) with Kruskal- Wallis test followed by Dunn test.

SEM examination

Stages 3 and 4 of bio-absorption (the most frequent stages observed in the present clinical study) were evaluated under SEM in order to analyze the manner of degradation. Unused PLLA screws had a compact appearance with an outer homogeneous surface (Fig. 4A,B,F). The differences in appearance between the unused screws and the fragments of screw removed from the patients are very evident both in those parts in contact with the biological environment and in the deeper seated ones. All samples analyzed appeared to be characterized by a dense, heterogeneous and globular outer layer; some samples were characterized by small pores on the surface, thus small and irregular cavities were detectable (Fig. 4G,H). Surface irregularities increased from stage 3 to 4 in parallel to the appearance of fibrillar structures (Fig. 4G,H). The transverse cross-sections revealed that inner fragments of the screws removed from patients (not in direct contact with the

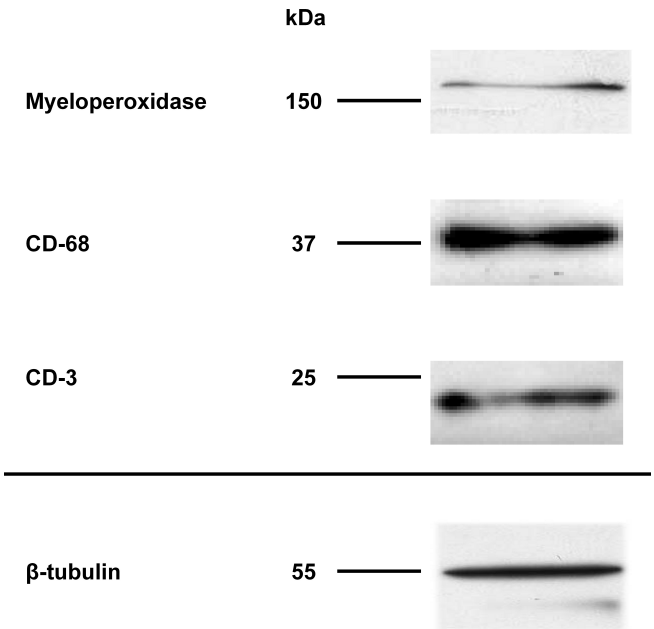


Fig. 3. Western Blot of inflammatory cell specific markers: CD68 for macrophages, CD3 for lymphocytes and Myeloperoxidase for neutrophils. Specimens obtained from soft tissues surrounding the screws after their removal. Anti-CD68 antibody recognizes a single intense band at the molecular weight of 37kDa; CD3, stands for 25 kDa band, appears very slightly; the band corresponding to Myeloperoxidase, at 150kDa, is poorly evident. Beta-tubulin is used as loading control.

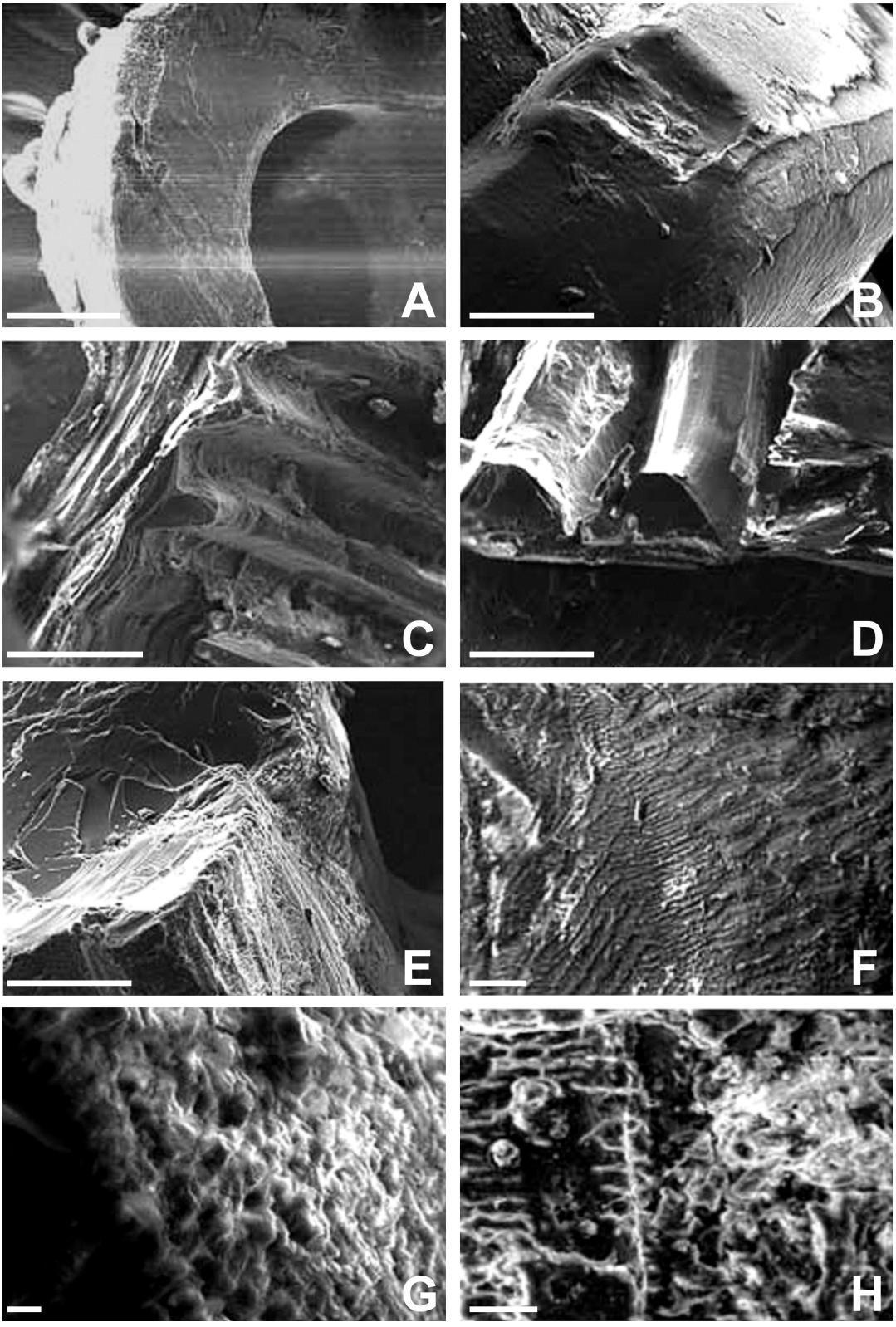


Fig. 4. Scanning electron micrographs showing the different aspect of original unused (**A, B, F**) versus removed (**C, D, E, G, H**) screws. Note in **C, D** (stage 3) breakings in the surface (arrows) inducing the formation of hollowed areas (visible in **G and H**). **E.** Screw remnant in stage 4. **F, G, H.** Enlargement of three screw remnant surfaces showing irregularities that increase in parallel to the stage of bio-absorption (**F**: unused; **G**: stage 3-44 months; **H**: stage 4-60 months). Scale bars: A-E, 0.5 μm; F, 5 μm; G, H, 50 μm.

tissue) were characterized by significant changes in the surface (Fig. 4E), when compared to that of the unused screws (Fig. 4A,B), and although at low magnification the surface areas of removed and unused screws have the same compact appearance, at higher magnification an irregular globular structure was visible close to the polymer layers which appeared separated and cracked. Moreover, as the degradation process progressed, in the majority of the devices, the transformation into hollowed structures was more evident and related to the elapsed time (Fig. 4C,D,G,H).

Discussion

The authors wish to underline that this is the first paper in which both histological analysis and bio-molecular evaluations have been performed in parallel to clinical and radiological outcomes of flat footed patients surgically treated with bioabsorbable PLLA screws.

The first point to discuss is the adequacy of the PLLA expansion endorthesis, used for healing in pediatric flat foot, to solve the problem. The data here reported showed that the deformity correction was satisfactory almost for the totality of patients (>80%). On the basis of MRI observations from patients treated with endorthesis, it can be stated that this material is efficiently bio-absorbed; however the bio-absorption period is long and often longer than expected, considering the indications in the literature by Giannini et al. (2003), which report a complete bio-absorption of PLLA four/five years after surgery. In the present study, the follow-up was widely variable because each patient was reviewed from the clinical point of view after three years, for the first time, and successively at different times, depending on the individual response to the discomfort due to incomplete screw bio-absorption. Moreover, it has to be considered that the examined population mainly includes children, whose motor activity and individual susceptibility to pain are very different: this fact has made the period of clinical assessment very variable. Both the *in vivo* observations and the morphological characterization of the fragments removed indicate that not less than 3 years after implantation (stages 3-4 of the bio-absorption) the screws degraded with a typical degradation mechanism, which involves both the surface and the interior of the device; the slow bio-absorption could depend on the difficulty of the small degradation products of PLLA to diffuse through the mass of the device. This particular effect could be due to different reasons, among which the polymer packaging performed during the fabrication process in order to obtain screws with suitable mechanical properties that make it possible to maintain adequate and lasting stability for healing. Also, the biological conditions of the site of implantation should have been considered; in fact, the external part of the sinus tarsi did not have the appropriate characteristics that could allow the optimal bio-absorption of the device, because of both its “low cellularity” (presence of adipose cells, loose connective tissue, poorly enzymatic activity) and the unsuitable chemical-physical environment. This is in line with some findings observed during the examination of pathological tissues removed from patients with *sinus tarsi syndrome*, which include chronic inflammatory changes, like fat necrosis, fibrosis and synovial cysts, all conditions consequent to bad vascularisation (Taillard et al., 1981; Lee et al., 2008).

The peculiar surgical condition shown in the present paper (i.e. the implant of PLLA device through the sinus tarsi) was completely different with respect to the classical cases in which the screws are implanted inside the bone, following which the referred PLLA degradation time ranged between 3 and 5 years and the bio-absorption was completed after 7-9 years (Barber and Dockery, 2006; Petricca et al., 2006; Warden et al., 2008); this fact (i.e. the implant site) could justify the wider temporal range needed to achieve a total bio-absorption of our screws. In our surgical procedure although the screws were well seated inside the sinus tarsi (in some cases long after the implantation, viz one or two years later), the screws tended to lose their correct position and move externally from the sinus tarsi; this new incorrect position prevented complete bio-absorption. Another consideration, based on SEM evaluation, concerns both the dynamics and the pattern by which the bio-absorption of the expansion-screw takes place. In most of the examined cases, the bio-absorption was observed to occur mostly in the intermediate and the medial parts of the screw, which are the most mechanically stressed parts during the prono-supination movements of the subtalar joint; this condition interferes with the overall time of bio-absorption. However, notwithstanding the hollowed structure morphology, well visible under SEM in removed screws at stages 3 and 4, the screw/tissue interface widens, but this increase of the interfacing areas is not sufficient to develop a cellular amount appropriate to complete the bio-absorption of the screws.

Analysis of the regression curves indicates that the stage of bio-absorption of the screw is closely correlated with the persistence period of the device inside the patient’s foot, and apparently it is hardly influenced at all by the diameter of the screw, even though the most satisfactory results were obtained with 8 mm diameter-screws. Among the cases studied, 64.5 % of them (in which 8-mm screws were implanted) showed a bio-absorption greater than 75% (stage 4).

Given the small number of screws completely bio-absorbed (stage 5), it can be assumed that after a long period of time the not bio-absorbed screw remnants inside the sinus tarsi probably persist without inducing recurrent episodes of inflammation that would cause discomfort to the patients. This fact could depend on the individual variation in response to PLLA insertion as well as on general health conditions.

Another point to discuss is the inflammatory response evaluated in our samples, since the inflammatory reaction was observed especially when the

screw remnants were dispersed into the soft tissues. Our observations agree in showing that once a biomaterial is introduced into the body, a sequence of events occurs in the surrounding tissue and ultimately ends in the FBGCs at the tissue/material interface. Obviously, implantation of foreign materials within the body provokes an acute inflammation as a result of the tissue injury, although what is unclear and largely overlooked are the mechanisms by which this ordinary transient phenomenon fails to resolve, leading to the development of a chronic inflammation that is widely described as detrimental to biomaterial function (Anderson, 1988). While acute inflammation is characterized by locally increased blood volume and leukocyte extravasation, chronic inflammation is marked by the presence of biomaterial surface-adherent macrophages fused to form FBGCs (McNally and Anderson, 2002). The mechanisms which govern the fate between resolution of acute inflammation or progression towards chronic inflammation are of critical importance for biodegradable materials like "polylactic acid" because the FBGC response ensures a long-term mechanism to eliminate the foreign material from the body. Frequent recurrent acute episodes can increase the occurrence of chronic involvement, which in turn is crucial to permit complete biomaterial bio-absorption (Böstman and Pihlajamäki 2000a,b; Solheim et al., 2000). In order to explain this connection better, the data here reported, concerning both histo-morphologic observations and western blot analysis, showed that a high percentage of monocytes/macrophages was present in the soft tissue samples examined. In this regard, a panel of antibody was used: anti CD68, anti CD3 and anti myelo-peroxidase. CD 68 is a transmembrane glycoprotein that is highly expressed by human monocytes and tissue macrophages. CD3 is the most widely used marker of normal and neoplastic T lymphocytes. CD3 is expressed all along the line of differentiation/maturation of T lymphocytes and is therefore considered a PAN-T marker. Myeloperoxidase is a peroxidase enzyme mostly present in neutrophil granulocytes; it is a lysosomal protein stored in azurophilic granules of the neutrophils. The results obtained clearly show the occurrence of chronic rather than acute inflammation in removed screw specimens.

Since FBGCs are derived from the fusion of macrophage and dendritic cell precursors, the presence of such cells in the wound healing compartment may be of crucial importance in directing the tissue reaction to biomaterial (Brodbeck et al., 2002; Matheson et al., 2004).

Despite the fact that little data is available on how the implantation of biodegradable materials may result in a chronic inflammatory response, chronic inflammation is often attributed to the irritant effect of a local reduction in pH, due in turn to acidic polymeric degradation products (Agrawal and Athanasiou, 1997). Moreover, macrophage fusion is dependent on the chemical components of the biomaterial used (Jones et al., 2004). The fact that we found in our samples a large amount of FBGCs is in line with data reported by other authors on important factors involved in FBGC triggering, like appropriate fusion-inducing-stimuli and the array of the proteins adsorbed to the screw surface, enhancing the cell-biomaterial relationships (Helming and Gordon, 2007; Keselowsky et al., 2007). The persistence of foreign body reaction over 3 years after implantation, in our specimens, and the fact that it is present at the interface "tissue/device" suggest that the oxidation processes continue over a period of time, albeit at low levels. Both the methods used to analyse the cellular population (i.e. western blot and histomorphometric analysis) agree in showing that the amount of macrophage/FBGCs does not differ, whether the patients are grouped according to the diameter of the screws implanted or according to the radiological outcomes, thus indicating a similar response to the implanted biomaterials. By contrast, the amount of screw remnants substantially differ among patients considered "very good" and "fair" from the clinical viewpoint. However, a comparative analysis between the two groups based on screw diameter cannot be performed, due to the poor sample size. Probably the microenvironment surrounding the device, induced in the first months after implant, is crucial to the subsequent process of bio-absorption of the screw and presumably depends on the individual response to the presence of foreign bodies.

The revaluation of PLLA parameters, as well as the geometry of screws, could improve the balance between bio-degradability and structural integrity of the device, in order to ensure the clinical target, decreasing the number of undesired events (i.e. the need for surgical screw removal) that partly frustrates the use of PLLA biodegradable screws in the correction of the flat foot in childhood.

In conclusion, on the basis of both histological evaluations and clinical/radiological outcomes, the authors underline the need for a better knowledge of "cell/biomaterial" interactions involved in the biological responses to implanted bio-materials to make screw devices more suitable in their application, although in our cases only a small cohort of patients needed screw removal.

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