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A pilot study of circulating levels of TGF-β1 and TGF-β2 as biomarkers of bone healing in patients with non-hypertrophic pseudoarthrosis of long bones

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ABSTRACT

Background: Pseudoarthrosis or non-union is a complication with an incidence of 5–10% of bone fractures, most frequently located in the diaphysis of long bones. The management of this complication is addressed by means of complex surgical procedures and is a concern for orthopaedic and trauma surgeons nowadays. The use of biomarkers for diagnosing patients at risk of non-union would help us to establish special measures for early corrective treatment.

Methods: Prospective exploratory pilot study with a cohort of 20 patients diagnosed of non-hypertrophic pseudoarthrosis of long bones who were treated surgically with either autologous bone graft or a Tissue Engineering Product composed of bone marrow-derived Mesenchymal Stromal Cells. Patients were followed for 12 months and plasma blood samples were obtained to determine circulating levels of Transforming Growth Factor Beta 1 and Beta 2 (TGF-β1 and TGF-β2, respectively) at inclusion, and at 1 week, 2 weeks, and months 1, 2, 3, 6 and 12 after surgery. Radiological bone healing was evaluated by the Tomographic Union Score (TUS).

Results: Basal levels of TGF-β1 and TGF-β2 were determined in the twenty patients (26,702 ± 14,537 pg/mL and 307.8 ± 83.1 pg/mL, respectively). Three of them withdrew from the study, so complete follow-up was conducted on 17 patients (9 successfully healed vs. 8 that did not heal). Statistically significant differences between the bone healing group and the non-union group were found at month 12 for both TGF-β1 ($p = 0.005$) and TGF-β2 ($p = 0.02$).

Conclusions: TGF-β1 and TGF-β2 are biomarkers that correlate with clinical evidence of bone regeneration and may be used to monitor patients, although early predictive value after intervention needs to be further studied in combination with other molecules.

1. Introduction

Pseudoarthrosis is a serious complication with 5–10% incidence rates of fractures, requiring complex surgical procedures (Tzioupis and Giannoudis, 2007; Ekegren et al., 2018). It is frequently located on the diaphysis of long bones, being the femur, tibia and humerus the most affected ones. The clinical presentation of pseudoarthrosis include pain from the fracture site and functional impairment. Two types of pseudoarthrosis have been defined, namely: 1) hyperthrophic (due to mechanical etiology), in which bone generation is observed but consolidation is not achieved due to excessive mobility in the fracture site; and 2) non-hypertrophic, in which proper mechanical stability is

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not sufficient to ensure consolidation due to a biological deficiency resulting from underlying pathologies or treatments (i.e. infection, diabetes, vascular disease, steroids). Surgical treatment of pseudoarthrosis provides: 1) stability at the fracture site in hypertrophic pseudoarthrosis and 2) biological stimuli (being iliac crest the current gold standard) in non-hypertrophic in addition to mechanical stabilisation where required. The need for replacing implants is assessed either 1) by imaging techniques if signs of osteolysis are observed surrounding the implant or 2) intraoperatively, if mobility of the osteosynthesis materials is observed. Strikingly, the diagnosis of pseudoarthrosis is still based on clinical, chronological and radiological criteria nowadays. Classically, pseudoarthrosis has been defined as the absence of bone healing at 9 months, although some authors recommend shortening patient monitoring to 6 months, if radiological signs of bone callus formation are not observed in three consecutive monthly radiological controls (Fisher et al., 2019). In any case, this is a considerably long waiting time that A) prolongs discomfort and dissatisfaction in patients, and B) impacts in health and social costs. Only in the UK, for instance, hospital costs attributed to the treatment of bone non-union has been quantified between 7000 and 79,000 pounds, on top of out-of-hospital and social health costs (Ekegren et al., 2018).

Molecular tools offer new opportunities for improving diagnostics and predictive medicine. This line of research is based on the detection of molecules involved in bone regeneration, either in peripheral blood or urine, which are used as biomarkers for monitoring the bone healing process (Chaverri and Vives, 2017; Pountos et al., 2013). Importantly, the use of a biomarker may be useful for patients at risk of non-union by assisting orthopaedists to establish special measures for early treatment to correct this situation. Human research on this topic is limited and methodologically poor, mainly based on cross-sectional and retrospective studies, with small sample size and inconclusive outcomes (Chaverri and Vives, 2017; Pountos et al., 2013; Sousa et al., 2015; Tatsuyama et al., 2000; Hankenson et al., 2014). At present, Transforming Growth Factor-Beta 1 (TGF-β1) is one of the most studied molecule in humans (Zimmermann et al., 2005; Zimmermann et al., 2007; Sarahrudi et al., 2011; Wang et al., 2009).

Transforming Growth Factor B (TGF-β) is a family of proteins secreted to the extracellular space to stimulate cell growth and replication. Three isoforms have been described: TGF-β1, TGF-β2 and TGF-β3 sharing 60–80% homology of their sequence. They have been related to the stimulation of cell replication, cartilage and bone formation, and fibrosis (Patil et al., 2011). Interestingly, most of the total TGF-β found in plasma corresponds to the TGF-β1 isoform, while TGF-β2 and TGF-β3 are found in amounts less than 5% (Wakefield et al., 1995).

TGF-β1 is a regulatory protein that is known to play an important role in bone healing and remodelling, promoting the proliferation and differentiation of mesenchymal cells, production of extracellular matrix, cartilage formation, as well as in the chemotaxis of bone cells (Patil et al., 2011). Although TGF-β1 is produced by diverse types of cells, platelets are considered the largest reservoir of TGF-β1 in the body with a concentration 100 times higher than in other tissues (Patil et al., 2011). In addition to platelets, bone is the second location with the highest concentration of TGF-β1 and it is also found in other tissues such as myocardiocytes and hepatocytes, although at lower quantities (Patil et al., 2011).

To date, research on TGF-β1 focuses on the evaluation of circulating levels and behaviour profile from bone fracture to diagnosis of delayed union and/or pseudoarthrosis (Zimmermann et al., 2005; Zimmermann et al., 2007; Sarahrudi et al., 2011). There is only one study in pseudoarthrosis that evaluates the behaviour of TGF-β1 with a non-surgical treatment, that is shock waves (Wang et al., 2009). To our knowledge, there are no studies in the literature investigating the use of TGF-β2 as biomarker of non-union. For this reason, in the present work, we propose to advance in the study of biomarkers in bone healing by analysing their behaviour from established pseudoarthrosis once a surgical therapeutic plan has already been implemented. This done by following, monitoring and analysing a cohort of patients diagnosed of non-hypertrophic pseudoarthrosis of long bones surgically treated and further evaluated the differences of TGF-β1 and TGF-β2 levels in blood.

2. Materials and methods

2.1. Aims

This study aimed to follow, monitor and analyse a cohort of patients diagnosed of non-hypertrophic pseudoarthrosis of long bones surgically treated and further evaluate the differences of TGF-β1 and TGF-β2 levels in blood according to: 1) the outcomes (that is bone healing or failure) (H_0 = no statistical differences between levels of biomarkers and treatment); 2) the assigned treatment (that is Tissue Engineering Product, TEP; or iliac crest autograft); and 3) follow up time, so that the predictive value could be assessed (H_0 = no statistical differences between levels of biomarkers and outcomes in the first 6 months).

2.2. Design and study participants

An exploratory prospective pilot study was carried out in a cohort of patients diagnosed with non-hypertrophic long-bone pseudoarthrosis, who were included in a prospective, single-centre, open label, randomised Phase IIa clinical trial with blind outcome assessment in which 20 patients aged 18 to 65 years affected with non-hypertrophic long-bone metaphyseal/diaphyseal pseudoarthrosis were selected (EudraCT No. 2013-005025-23; ClinicalTrials.gov Id. NCT02230514). The study was carried out between 2014 and 2019 at ASEPEYO Sant Cugat Hospital, where the patients were recruited, treated and followed up, and the samples were stored and analysed at Banc de Sang i Teixits (Barcelona, Spain). No previous sample size calculation was made because of the pilot nature of this study. Patients were randomised to either one of the two study treatments described next: 1) Treatment A (experimental): mechanical stabilisation (if required) associated with a Tissue Engineering Product (TEP composed of ex vivo expanded autologous Mesenchymal Stromal Cells (MSC) loaded onto allogeneic cancellous bone graft described previously (Prat et al., 2018; García de Frutos et al., 2020) and prepared in accordance with current Good Manufacturing Practices as reported elsewhere (Codinach et al., 2016; Vives et al., 2021; García-Muñoz and Vives, 2021); and 2) Treatment B (control): mechanical stabilisation, if required, associated with autologous iliac crest graft (gold standard) (Figs. 1 and 2). After treatment, patients were followed for a period of 12 months with monthly control radiographs (Rx) until 6 months and then at 9 and 12 months, and computerised tomography (CT) at 12 months. Blood tests were conducted at 1 week, 2 weeks, 1 month, 2 months, 3 months, 6 months, and 12 months. Surgery was carried out by the same team of 3 orthopaedic surgeons in all cases. All patients received the same post-operative analgesia and antithrombotic prophylaxis protocol.

Inclusion criteria: patients 18–65 years old with non-hypertrophic pseudoarthrosis of long bones diagnosed by Computerised Tomography (CT) scan at 9 months. Signature of informed written consent. Exclusion criteria: active septic process, active smoking, diabetes mellitus, peripheral arterial vascular disease, positive serology for HIV, Hepatitis B, Hepatitis C or Syphilis, pregnancy, congenital bone diseases, metabolic bone disease associated with primary or secondary hypoparathyroidism, neoplastic disease detected in the last five years.

This study was approved by the Clinical Research Ethics Committee (CEIC idcsalud, Catalunya) and conducted in accordance with the declaration of Helsinki. All patients gave their informed written consent for participation in the study.

2.3. Collection, handling and storage of blood samples

Peripheral blood samples were taken at the following time points: Inclusion, 1 week, 2 weeks, 1 month, 2 months, 3 months, 6 months, and

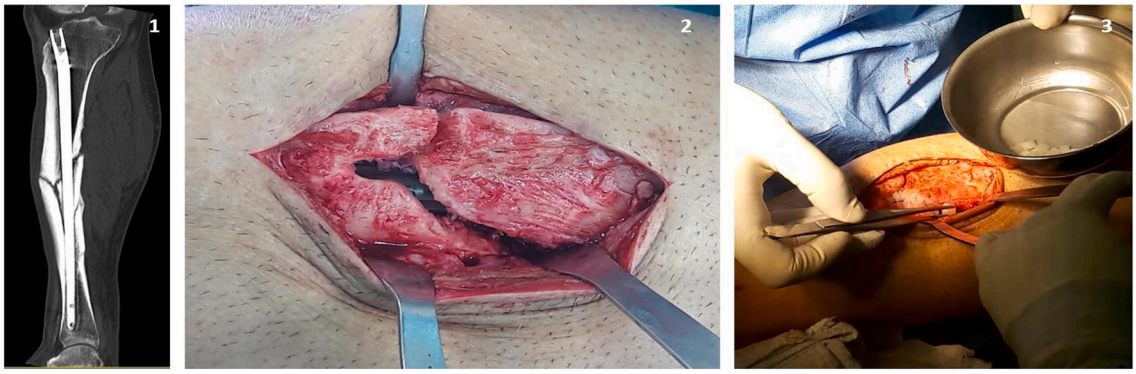

Fig. 1. Patient no. 19 (experimental group). This is a 60-year-old male affected by non-hypertrophic diaphyseal pseudoarthrosis of tibia with endomedullary nail diagnosed by CT scan 9 months after the fracture (1). Treatment consisted on the removal of the endomedullary nail, fixation with a new nail, debridement of the pseudoarthrosis site and implantation of the Tissue Engineering Product. In (2), the pseudoarthrosis focus just debrided after the replacement of the tibia nail. In (3), application of cubes of the Tissue Engineering Product consisting of bone matrix loaded with autologous bone marrow-derived mesenchymal stromal cells.

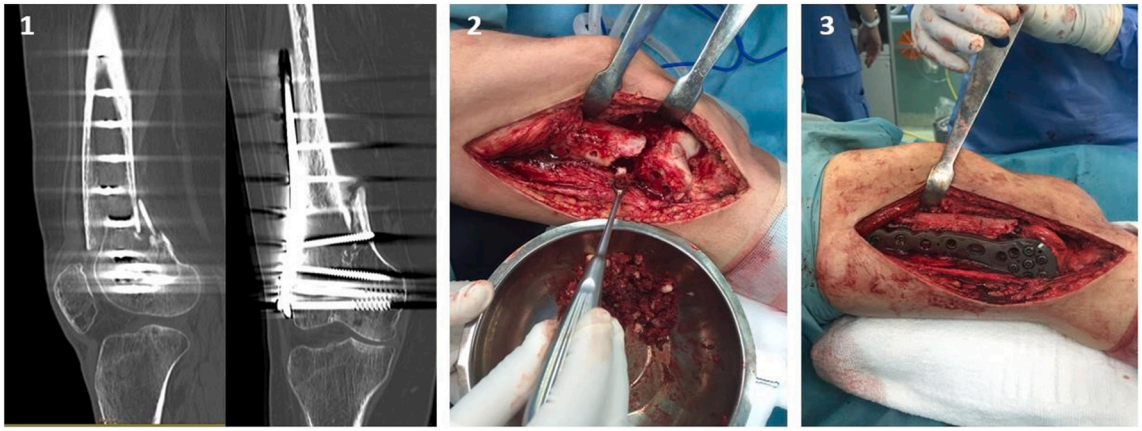

Fig. 2. Patient no. 7 (control group). This is a 44-year-old woman suffering with non-hypertrophic pseudoarthrosis of the femur treated with a plate and diagnosed by CT scan 9 months after fracture (1). Treatment was: plate removal, debridement of the pseudoarthrosis site and implantation of autologous bone graft (2). Intraoperative picture of the pseudoarthrosis focus already curetted and implantation of autologous iliac crest bone graft. In (3), osteosynthesis with specific lateral distal femur plate.

12 months. Blood collections were performed in the morning with the patient fasting. Recommendations made by Zhao and collaborators were followed for handling the samples (Zhao et al., 2012). Briefly, peripheral blood sample was obtained by venepuncture and collected in a tube with anticoagulant (1.6 mg EDTA/mL). It was immediately centrifuged at 2500*g* for 10 min at 4 °C. After centrifugation, 1 mL of the supernatant (plasma) was collected and stored at −80 °C until use. After thawing and prior to the final analysis, samples were centrifuged at 10,000*g* for 15 min at 4 °C. The extraction, handling and centrifugation of the sample was always carried out by the same team of laboratory staff who were previously trained in the protocol.

2.4. Evaluation of bone healing

The evaluation of bone healing was carried out by the same researcher, a blinded radiologist, using a CT scan at 12 months and applying the Tomographic Union Score (TUS). A TUS score greater or equal to 11 was established to determine radiological healing (Leow et al., 2016; Litrenta et al., 2015; Perlepe et al., 2013).

2.5. Sample analysis

The samples were analysed by multiplex luminometric assay using xMap Bio Plex Pro® TGFB immunoassay technology (Bio-Rad Laboratories, Hercules, CA, USA) using the Luminex 100IS analyser (Luminex Corp. Austin. TX, USA) following the manufacturer's recommendations (Grau-Vorster et al., 2019). Data analysis was performed using Bioplex Manager v6.1 software (Bio-Rad Laboratories Inc.). The amount of TGF-β1 and TGF-β2 in plasma was quantified in pg/mL. All samples were analysed by the same team in the time period comprised between December 2018 and January 2019.

2.6. Statistical analysis

Non-parametric U Mann-Whitney test for unpaired samples was used to compare the values of TGF-β1 and TGF-β2 between the two groups under study (bone healing vs no bone healing and TEP vs iliac crest graft) at different time points. Data are presented as mean ± standard deviation. The statistical analysis was carried out using the SPSS 15.0 software package for Windows (SPSS Inc., Chicago, IL, USA) and the significance level was set at $^{*}p < 0.05$.

3. Results

Twenty (20) patients were enrolled: 17 men (85%) and 3 women (15%), with a mean age of 47.9 ± 9.4 years old (men: 24 to 60; women: 44 to 59). Most relevant demographic and clinical characteristics of patients are described in Table 1. Initial treatment of the fracture was either endomedullary (EM) nailing in 55% of cases or using a plate (45% of cases). To treat pseudoarthrosis, hardware was replaced in 10 cases

Table 1
Clinical demographic characteristics of patients.

ID	Sex	Age	Bone	AO classification[1]	Initial treatment	Biological stimulus	Hardware replacement	Adverse events
1	M	55	Humerus	12 A1	Nail	Autograft	No	No
2	M	47	Humerus	12 A1	Nail	TEP	Yes (plate)	No
3	M	51	Femur	33 A3	Plate	TEP	Yes (nail)	No
4	M	40	Femur	33 C2	Plate	Autograft	Yes (nail)	Infection[2]
5	M	36	Humerus	12 B2	Nail	TEP	No	No
6	M	59	Cubitus	2 U2A	Plate	Autograft	No	No
7	F	44	Femur	33 B2	Plate	Autograft	Yes (plate)	No
8	M	42	Tibia	41 A2	Plate	TEP	No	Infection[2]
9	M	38	Humerus	12 A3C	Plate	TEP	No	No
10	M	52	Tibia	42 A3B	Plate	Self-excluded	N/A	Self-excluded
11	M	44	Tibia	43 A2.3	Plate	Autograft	Yes (plate)	No
12	M	55	Tibia	41 C1	Plate	Autograft	Yes (plate)	No
13	F	59	Humerus	12 A2	Nail	Autograft	Yes (nail)	No
14	M	51	Tibia	42 C3	Nail	Autograft	Yes (plate)	No
15	M	24	Femur	32 B2	Nail	TEP	No	No
16	F	47	Humerus	12 A3C	Nail	TEP	No	No
17	M	50	Tibia	42 B2	Nail	Autograft	No	No
18	M	42	Tibia	42 B2	Nail	TEP	No	No
19	M	60	Tibia	42 C2	Nail	TEP	Yes (nail)	No
20	M	62	Tibia	42 C2	Nail	Autograft	Yes (plate)	No

M = male; F = female; N/A = not applicable.
[1] Type of fracture is based on the AO Foundation classification (Meinberg et al., 2018).
[2] Excluded; TEP: Tissue Engineering Product composed of ex vivo expanded autologous Mesenchymal Stromal Cells loaded onto allogeneic cancellous bone graft).

(52.6%) and the implant was retained in 9 cases (47.4%). In 10 cases, an autologous iliac crest graft was implanted (52.6%) and in 9 cases a TEP was used (47.4%) according to clinical trial protocol.

Two patients withdrew the study for adverse events, infection in both cases, while another patient voluntarily self-excluded. These three patients were not included in the statistical analysis. Thus, 17 patients were finally studied. From a total of 136 blood samples, 96.3% of them were analysed, being 3.67% missing values (5 samples: 3 due to default of appearance of the patient and 2 technical errors leading to missing the samples).

3.1. Analysis based on bone healing

The cohort was divided into 2 groups: healing and non-healing. A total of nine patients healed after 12 months vs 8, who did not heal within that time. Remarkably, statistically significant differences were found in month 12 for TGF-β1 ($p = 0.005$) and TGF-β2 ($p = 0.02$) (Fig. 3A).

3.2. Analysis based on the treatment

In order to evaluate the influence of the type of treatment on the

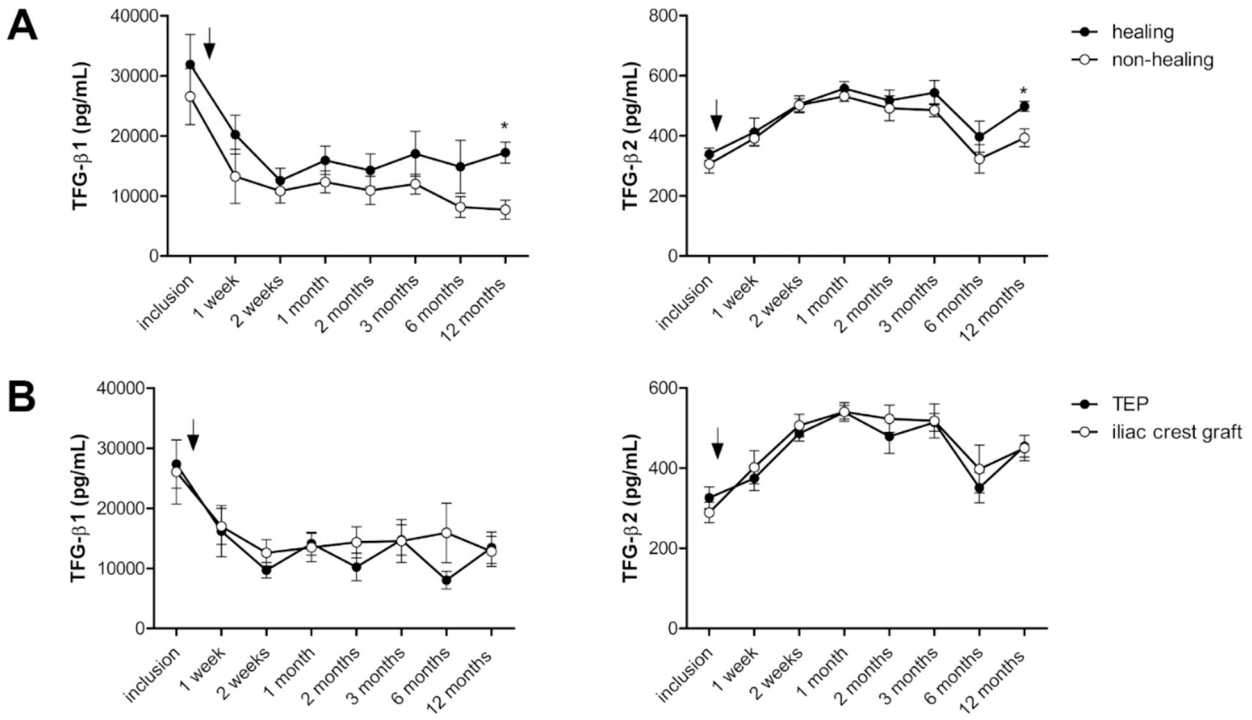

Fig. 3. Circulating levels of TGF-β1 and TGF-β2. Levels of TGF-β1 and TGF-β2 are shown in the different time points measured according to bone healing (A) and treatment (B). The arrows indicate the time of surgery. * shows significant differences between the two groups (TGF-β1: $P = 0.005$; and TGF-β2: $P = 0.02$). TEP = Tissue Engineering Product.

values of TGF-β1 and TGF-β2, the cohort was divided into two groups (TEP or iliac crest autograft). No statistically significant differences were found for TGF-β1 or TGF-β2 in any of the time points assessed (Fig. 3B).

3.3. Levels of TGF-β1 and TGF-β2

Total mean values of TGF-β1 in the bone healing group were 17,724 ± 5728 pg/mL. The total mean values of TGF-β1 in the non-healing group were 13,730 ± 5873 pg/mL. Mean baseline value of TGF-β1 at inclusion time (from all 20 patients included) was 26,702.4 ± 14,537 pg/mL (range: 5863.25–60,636.28).

Total mean values of TGF-β2 in the bone healing group were 449.6 ± 98.5 pg/mL. The total mean values of TGF-β2 in the non-healing group were 417 ± 87 pg/mL. Mean baseline value of TGF-β2 at inclusion time (from all 20 patients included) was 307.8 ± 83.1 pg/mL (range: 124.05–435.58).

4. Discussion

4.1. Strengths and weaknesses

To our knowledge, this is the first study to assess the behaviour of two biomarkers, TGF-β1 and TGF-β2, in non-hypertrophic pseudoarthrosis of long bones after surgical treatment. Other previous studies assessed several biomarkers but mainly in fractures (Zimmermann et al., 2005; Zimmermann et al., 2007; Grgurevic et al., 2007; Ohishi et al., 2008; Sarahrudi et al., 2010; Sarahrudi et al., 2009; Singh et al., 2013), aseptic loosening in hip arthroplasties (Goebel et al., 2009) and orthopaedic surgical treatments in children (Granchi et al., 2013).

Another study evaluated TGF-β1 and several biomarkers in pseudoarthrosis after shock wave therapy treatment (Wang et al., 2009). These authors included patients with hypertrophic and non-hypertrophic pseudoarthrosis. In contrast, in our study we only included patients suffering from non-hypertrophic pseudoarthrosis. In a recently published paper, Granchi and collaborators assessed different biomarkers in aseptic pseudoarthrosis surgically treated with a tissue engineering product in the context of a clinical trial, with a study design similar to ours (namely: C-Propetide of Type I Procollagen, CICP; C-terminal telopeptide of type I collagen, CTX; Bone isoform of Alkaline Phosphatase, BAP; Nterminal/midregion fragment of Osteocalcin, N-Mid OC; Osteoprotegerin, OPG; Receptor Activator of Nuclear Factor-κB ligand, RANKL) (Granchi et al., 2019).

TGF-β1 has been studied in cohorts of patients with long bone fractures that evolve into pseudoarthrosis (Zimmermann et al., 2005; Zimmermann et al., 2007; Sarahrudi et al., 2011). Sarahrudi and collaborators included hypertrophic and non-hypertrophic pseudoarthroses although they finally performed a statistical analysis excluding the hypertrophic ones (Sarahrudi et al., 2011). Of note, our study started at the moment of the diagnosis of the pseudoarthrosis, whereas the previous work actually terminated at that point potentially explaining the different profiles of TGF-β1 levels observed.

A strength of our study is that the sample is previously selected using strict inclusion/exclusion criteria so that risk factors that may influence in bone healing are minimized (i.e. smoking, diabetes, age, anti-osteoporosis drugs) (Kaiser et al., 2012). Although other studies tried to control these factors, no such strict criteria were used (Zimmermann et al., 2005; Zimmermann et al., 2007; Sarahrudi et al., 2011; Wang et al., 2009).

One of the greatest strengths of the present study is the handling process of blood samples. In this sense, the study published by Zhao and collaborators in 2012 marked a turning point in the analysis of TGF-β1 blood levels, showing the impact of an incorrect sample handling process and how this can significantly alter the results (Zhao et al., 2012). This can question or even invalidate certain studies carried out previously. Studies published before 2012 do not specify in detail how the sample was handled. Our research takes into account the recommendations made by Zhao and collaborators and, remarkably, the absolute values we found are lower than those reported in other studies (Table 2). This observation is consistent with a robust handling sample process protocol followed in our study, provided that temperature and centrifugation are decisive to avoid platelet degranulation and sample contamination by TGF-β1 from platelets. The lack of control of these parameters in previous studies may have resulted in higher mean values.

Although the Enzyme-Linked Immunosorbent Assay (ELISA) is the most widely used method and could be therefore considered as the "gold standard" to measure levels of cytokines and growth factors, multiplex technology offers similar sensitivity and specificity values compared to ELISA (Elshal and McCoy, 2006; Khan et al., 2004; Hernández Ramírez and Cabiedes, 2010). Moreover, multiplex technology allows: A) convenient analysis of several molecules at the same time, as we did in our case (TGF-β1 and TGF-β2); B) it is fast and reproducible; and C) it is especially useful with small samples.

On the other hand, we acknowledge a weak point of our study regarding the small sample size completing the study (n = 17), due to the pilot nature of this study. However, similar studies found in the literature share the same weakness (as summarized in Table 2). It should be noted that pseudoarthrosis is not a prevalent pathology (especially in our case, focusing on non-hypertrophic pseudoarthrosis with restrictive inclusion criteria) and large samples are extremely difficult to recruit, in our case, patient recruitment took 4 years in one single centre. Alternatively, multi-centre studies, as described by Granchi and collaborators, may be a solution for future research (Granchi et al., 2019). We also observed a gender bias in the treated population (17 male vs. 3 female), although this was probably due to the casuistry, provided that no evidence of gender-dependency has been described in the literature for bone healing.

4.2. Values and behaviour of biomarkers

The mean baseline values at inclusion time of TGF-β1 (26,702.4 ± 14,537 pg/mL) and TGF-β2 (307.8 ± 83.1 pg/mL) in 20 patients with non-hypertrophic pseudoarthrosis can be considered the first described in the literature. Wang and collaborators measured baseline levels of TGF-β1 in patients with pseudoarthrosis (46,275 ± 11,175 pg/mL) but it should be noted that these authors did not differentiate between hypertrophic and non-hypertrophic pseudoarthrosis (Zhao et al., 2012). Their sample size is lower than our study (12 vs 20 subjects), and blood sample handling process is not sufficiently described. Furthermore, the study was carried out in 2009, before Zhao and collaborators in 2012 demonstrated the influence of blood handling process in the values of TGF-β1. This could be the reason why their values differ so much from ours (Table 2).

TGF-β1 indicated bone formation according to the outcomes of our study. TGF-β1 values were always higher in the bone healing group, although significant differences were found only at month 12. This could be explained because of the small sample size. Therefore we cannot state at this point that TGF-β1 is a biomarker for early detection of non-union. In our opinion, predictive potential of TGF-β1 could have been considered only if significance were observed in the first 6 months.

The highest levels of TGF-β1 were found at the beginning of the study, when the fracture focus displays non-union, high instability and an elevated inflammatory pattern given the continuous failed attempts of bone repair. After surgery, TGF-β1 values drop, which we think is logical, because debridement and stability could break the chronic inflammation state in the zone.

Similar to TGF-β1, levels of TGF-β2 are higher in the healing group, but statistical significance is found only at month 12. Interestingly, unlike TGF-β1, TGF-β2 shows its lower levels at the beginning of the study, in an established pseudoarthrosis scenario. It is known that the 3 isoforms of TGF-β are essential in bone formation, although the expression patterns, as well as the secretion times, differ and are unknown, especially in humans in an established pseudoarthrosis

Table 2
Values of TGF-β1 in the different human studies on long bones.

Pathology	Ref.	Method	Inclusion		1 week		2 weeks		1 month		2 months		3 months		6 months		12 months	
			H	NH	H	NH	H	NH	H	NH	H	NH	H	NH	H	NH	H	NH
Pseudoarthrosis	This study (9H vs 8NH)	Multiplex	31,895 ± 15,002	26,537 ± 13,262	20,203 ± −9724	13,249 ± 12,684	12,539 ± 6242	10,803 ± 5595	15,919 ± 7076	12,324 ± 5141	14,246 ± 7747	10,929 ± 6268	17,016 ± 11,235	11,969 ± 4658	14,850 ± 12,482	8152 ± 4266	17,203 ± 4916	7716 ± 3844
	Wang et al. (2009) (9H vs 3NH)	ELISA	50,722 ± 10,807	46,275 ± 11,175	n.d.	n.d.	n.d.	n.d.	60,986 ± 13,661	49,337 ± 8132	n.d.	n.d.	54,250 ± 15,680	46,791 ± 11,160	48,590 ± 13,457	46,297 ± 12,437	47,156 ± 8534	35,488 ± 13,441
Fractures	Zimmermann et al. (2005) (10H vs 10NH)	ELISA	n.d.	n.d.	58,460 ± 14,790	46,330 ± 12,580	66,000	59,000 *	58,000*	45,000*	54,000*	46,000*	53,000*	48,000*	51,000*	51,000*	n.d.	52,000*
	Sarahrudi et al. (2011) (9H vs 9NH)	ELISA	14,171 ± 5642	14,171 ± 5642	29,178 ± 1364	27,339 ± 2973	36,334 ± 1688	34,265 ± 4337	33,000*	27,939 ± 3327	31,932 ± 1397	35,000*	32,500*	32,500*	35,267 ± 2220	33,500*	n.d.	n.d.

H = Healed; NH = Non-Healed; n.d. = time points not analysed in the study. The values that have been described in the studies are in absolute mean values with standard deviations.
* Values obtained from graphs shown in the different papers without standard deviations

situation. Additional studies are needed to analyse the behaviour of the three isoforms together in order to understand patterns of their secretion as well as their synchronisation. Recently, Ripamonti and collaborators have shown that it is possible to generate the same amount of ectopic bone by injecting either high doses of TGF-β3 into the stomach's mice or injecting low doses of TGF-β1 and TGF-β3 combined, which would support the synergy concept of the three isoforms, and therefore the idea of studying them as a whole and not as isolated entities (Ripamonti et al., 2016).

It has been shown in animal studies that TGF-β1 works by stimulating osteoblast migration and differentiation of osteoblasts and osteoclasts in early stages, as well as in the period of later bone remodelling (Takeyama et al., 2016; Kalinichenko et al., 2017; López-Fernández et al., 2020). Indeed, we have previously reported the involvement of TGF-β1 in osteogenic induction of multipotent Mesenchymal Stromal Cells (MSC) in vitro (Cabrera-Pérez et al., 2019). These data would support that the behaviour of TGF-β2 shows an elevation up to month 1 after surgery, and then a slow drop to rise again until month 12.

Provide that the null hypothesis in our study was true regarding the predictive value of TGF-β1/2 levels at month 6, we hypothesize that early detection of non-union should be based not only in the use of a single biomarker, but the combination of several of them. We strongly believe that this would be something to bear in mind for future research in this field.

4.3. *TGF-β1 and TGF-β2 in healthy subjects and in other pathologies*

Nowadays, the members of the TGF-β family of proteins are under investigation to demonstrate their potential as biomarkers in several pathologies: cancer (i.e. breast, pancreatic adenocarcinoma, prolactinomas, oral carcinomas), liver damage, arteriosclerosis, among others.

Remarkably, the established basal levels of TGF-β proteins in healthy people have changed over time due to the improvement of sample handling and analysis techniques. Letterio and collaborators were the first to describe in 1995 the TGF-β values in a sample of 42 healthy subjects by ELISA obtaining TGF-β1 mean values of 4100 ± 200 pg/mL and TGF-β2 < 200 pg/mL (Wakefield et al., 1995). Sarahrudi and collaborators in 2011 performed their study with 33 healthy volunteers by ELISA showing TGF-β1 levels in plasma of 29,735.3 ± 1328.4 pg/mL (Sarahrudi et al., 2011).

However, the most reliable values are those described after 2012, when Zhao and collaborators defined new standards of the blood handling process (Zhao et al., 2012). For example, in 2013, Elenkova and collaborators showed TGF-β1 values in healthy patient controls by ELISA technique (n = 48) of 15,800 ± 7200 pg/mL (Elenkova et al., 2013). In 2019, Martin-Gonzalez and collaborators reported the values of this biomarker in a sample of 34 healthy individuals, being 14,124 ± 8787 pg/mL (Martín-González et al., 2019).

It is interesting to compare the total mean values of TGF-β1 in our study (17,724 ± 5728 pg/mL in the healing group, and 13,730 ± 5873 pg/mL in the non-healing group) with other pathologies such as: alcoholic patients with liver disease (n = 79) 19,822 ± 12,282 pg/mL (Martín-González et al., 2019); patients with macroprolactinomas (n = 28) 23,900 ± 10,300 pg/mL (Elenkova et al., 2013); and patients with invasive prolactinomas (n = 29) 24,100 ± 9900 pg/mL (Elenkova et al., 2013). The values in our sample are close to those of healthy subjects as well as were not as high as those of patients with malignant and aggressive tumours, which confirms the "benignity" of the cellular processes involved in pseudoarthrosis, as a pathology.

The same statement can be extrapolated to TGF-β2, whose mean values ranged from 449.6 ± 98.5 pg/mL in the healing group to 417 ± 87 pg/mL in the non-healing group; while in pancreatic adenocarcinomas these values are two-fold higher (885.95 ± 143 pg/mL), as reported in 2019 by Vicente and collaborators using Multiplex technology in a sample of 14 patients (Vicente et al., 2019).

5. Conclusions

TGF-β1 and TGF-β2 are biomarkers that correlate with clinical evidence of bone regeneration at later stage of bone repair and may be used to monitor patients and diagnose non-union. At this time, however, the use of only these two biomarkers is not sufficient for early detection of bone healing failure after intervention. Therefore, translational clinical application of this study may require the combination of TGF-β molecules with other diagnostic techniques.

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Institutional review board statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Clinical Research Ethics Committee (CEIC idcsalud, Catalunya) (protocol XCEL-PSART-01, January 31st 2014).

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

CRediT authorship contribution statement

Daniel Chaverri: Conceptualization, Supervision, Investigation, Writing – original draft, Writing – review & editing. **Daniel Vivas:** Investigation, Writing – review & editing. **Santiago Gallardo-Villares:** Investigation, Writing – review & editing. **Fernando Granell-Escobar:** Investigation, Writing – review & editing. **Javier A. Pinto:** Investigation, Writing – review & editing. **Joaquim Vives:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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