Circulating endothelial cells and their progenitors in systemic lupus erythematosus and early rheumatoid arthritis patients

Javier Rodríguez-Carrio¹, Catuxa Prado¹, Banesa de Paz¹, Patricia López¹, Jesús Gómez², Mercedes Alperi-López³, Francisco J. Ballina-García³ and Ana Suárez¹

Abstract

Objective. The aim of this study was to investigate the endothelial progenitor cell population in SLE and early RA patients and its potential relationships with disease features and cytokine serum levels.

Methods. Endothelial progenitor cells (EPCs), mature EPCs (mEPCs) and endothelial cells (ECs) were measured in peripheral blood samples from 83 SLE and 85 early RA patients and 39 healthy controls by flow cytometry on the basis of CD34, VEGF receptor 2 and CD133 expression. Serum levels of IL-1β, IL-6, IL-8, IL-17, VEGF-A, IFN-α, TGF-β and GM-CSF were quantified by immunoassays. Clinical and immunological data were obtained by reviewing clinical histories.

Results. Circulating EPCs were increased in SLE but not in early RA patients associated with an enhanced CD34⁺ bone marrow-progenitor cell release but unrelated to disease features. The amount of mEPCs, however, was significantly higher in SLE patients presenting anti-SSA/SSB antibodies and/or malar rash, whereas the presence of specific autoantibodies was associated with EC counts in early RA and SLE patients. As expected, most cytokines tested were altered in both diseases but, interestingly, IFN-α levels, and to a lesser extent IL-6 and IL-1β, were associated with CD133 loss and increased mEPC number, whereas VEGF and TGF-β seem to exert an opposite effect.

Conclusion. Our results show that high IFN-α levels and/or the presence of disease-specific antibodies may identify a group of SLE patients with increased mEPC and EC counts, and consequently probably defective endothelial repair, thus supporting their use as surrogate biomarkers of endothelial damage and high cardiovascular risk.

Key words: systemic lupus erythematosus, rheumatoid arthritis, endothelial progenitor cells, vascular damage, autoantibodies, interferon-α.

Introduction

Several studies have demonstrated that most autoimmune patients have an increased cardiovascular risk, leading to high cardiovascular morbidity and mortality rates. However, the accelerated development of atherosclerosis observed in patients with SLE and RA cannot be fully accounted for by traditional Framingham risk factors [1], and it has been proposed that systemic inflammation and immune-mediated disease-related mechanisms could play a pivotal role in the increased cardiovascular risk [2], probably through adhesion molecules and pro-inflammatory cytokine overexpression [3]. New cardiovascular damage biomarkers are needed to improve the cardiovascular health of autoimmune patients.

Vascular endothelial injury, increased in autoinflammatory conditions, is the primary event in atherosclerosis and its regeneration involves endothelial progenitor cell (EPC) mobilization from the bone marrow and their recruitment...
into damaged tissue. EPCs are a heterogeneous population whose physiological role is to carry out vasculogenesis and vascular repair [4]. EPCs express endothelial [VEGF receptor-2 (VEGFR2)] and haematopoietic (CD34 and CD133) cell markers. Nevertheless, there is no consensus on the precise definition of EPC. During differentiation to mature EPCs (mEPCs), CD133 expression is lost and begins to express vascular endothelial (VE)-cadherin and von Willebrand factor [5]. Because of their potential role in vascular repair, the amount of circulating EPCs is considered a surrogate marker for vascular dysfunction and cardiovascular risk, and thus a promising tool in cell therapy for cardiovascular diseases, especially in connective tissue diseases [6].

Variations in the level of circulating EPCs were reported in different conditions affecting the vascular system. Several reports have shown that reduced levels or impaired EPC function correlated inversely with cardiovascular risk factors and cardiovascular outcomes [7, 8]. In the field of autoimmune diseases, contradictory results have been reported. Indeed, some studies suggested a lower EPC number in both SLE [9-13] and RA [14, 15], but conversely, others reported higher values [16, 17], no differences [18] or impaired function [19, 20]. Lack of standardized procedures is probably the main source of variability between studies [21].

Pro-angiogenic factors, such as VEGF, can mobilize EPCs and may potentiate their recruitment to the site of endothelial injury, whereas anti-angiogenic molecules exert an opposite effect. In fact, several pro-inflammatory and immunosuppressor cytokines, including IL-1β, IL-6, IL-8, IL-17A, TGF-β, GM-CSF and IFN-α, are involved in EPC mobilization, survival, recruitment or function. Given that most of them have been reported to be deregulated in autoimmune patients, these alterations could be a relevant immune-mediated mechanism involved in vascular damage and EPC dysfunction. In addition, it has been suggested that disease-specific autoantibodies also affect endothelial cells (ECs), causing inflammatory vascular damage and EC apoptosis [2, 22]. Because of the contradictory results reported with regard to circulating EPCs in autoimmune diseases, in this work we determined circulating ECs and their progenitors in SLE and RA patients, and evaluated the potential associations with disease features and serum levels of cytokines relevant for these autoimmune disorders.

Materials and methods

Patients and controls

The study group consisted of 83 SLE patients selected from the Asturian Register of Lupus [23], 85 patients with early RA consecutively recruited from the Early Arthritis Diagnosis outpatient clinic of the Hospital Central de Asturias and 39 sex- and age-matched healthy controls (HCs, 32 women and 7 men; mean age [6.0] 42.6 (11.3)), all of whom were of Caucasian origin. The ACR criteria were used for the diagnosis of RA and SLE. Information on clinical and immunological manifestations and therapies received was obtained by reviewing clinical histories. SLEDAI and DAS-28 were used to evaluate disease activity in SLE and RA patients. Informed consent was obtained from all participants, and the study was approved by the Regional Ethics Committee for Clinical Investigation from Hospital Universitario Central de Asturias (Oviedo), in compliance with the Declaration of Helsinki. None of the patients or controls had a history of coronary artery disease, myocardial infarction, cardiac insufficiency, statin therapy, cancer, diabetes or smoking habit.

EPC quantification

EPCs were analysed by FACS as described previously, following European League Against Rheumatism (EULAR) Scleroderma Trials and Research (EUSTAR) recommendations on EPC measurement [24]. Briefly, 100 μl of peripheral blood was pre-incubated with 10 μl of FcR blocking reagent (Miltenyi Biotech, Bergisch Gladbach, Germany). Then the cells were incubated with anti-VEGFR2-phycocerythrin (R&D Systems, Minneapolis, MN, USA), anti-CD34-FITC (BD Pharmingen, San José, CA, USA), and anti-CD133-allophycocyanin (Miltenyi Biotech) or with isotype-matched antibodies (BD Pharmingen). Labelled cells were analysed in a BD FACS Canto II flow cytometer with FACSDiva Software acquiring at least 100 000 events per sample. EPCs were identified as triple-positive cells for CD34/VEGFR2/CD133 in the lymphocyte gate. CD34+/VEGFR2+/CD133- and CD34+/VEGFR2+/CD133- were identified as mEPCs and ECs, respectively. Cell counts were expressed as the number of positive cells per 100 000 events in the lymphocyte gate.

Quantification of cytokine serum levels

Serum samples were stored at −80°C until cytokine measurements. VEGF-A165, IFN-α, GM-CSF, IL-1β, IL-6, IL-8 and IL-17A were quantified using a Cytometric Bead Array Flex Set (BD Biosciences, San José, CA, USA) and analysed in a BD FACS Canto II flow cytometer using FCAP Array software (BD Biosciences). For IL-1β, IL-6 and IL-17A, an Enhanced Sensitivity Flex Set was used. The assay sensitivity was 4.0 pg/ml for VEGF-A165, 1.5 pg/ml for IFN-α, 0.2 pg/ml for GM-CSF, 48.4 fg/ml for IL-1β, 68.4 fg/ml for IL-6, 1.2 pg/ml for IL-8 and 26.1 fg/ml for IL-17A. TGF-β1 was quantified using an ELISA kit (eBioscience, San Diego, CA, USA), according to the manufacturer’s instructions, with 5 ng/ml of detection limit.

Statistical analyses

All data were analysed with SPSS software (v.15.0) (IBM, Armonk, NY, USA). The Kolmogorov–Smirnov test was used to evaluate normality. The Mann–Whitney U test was used to compare differences between groups, and Spearman’s rank test was used to evaluate correlations. Data were expressed as median (interquartile range) unless stated otherwise. A P-value of <0.05 was considered statistically significant.
Results

Circulating CD34+, EPC and EC populations in SLE and RA patients

The presence of ECs and their progenitors in the peripheral circulation of 39 HC, 83 SLE and 85 early RA patients (23 recruited at diagnosis) was analysed by flow cytometry following the gating strategy shown in Fig. 1A. After selecting the lymphocyte population, CD34+ cells were gated and assessed for CD133 and VEGFR2 expression. Triple-positive cells were considered EPCs and CD34+VEGFR2+CD133− cells mEPCs. On the other hand, VEGFR2+ cells on the lymphocyte gate were selected and those lacking CD34 and CD133 expression were considered ECs detached from the endothelial wall.

Tables 1 and 2 show demographic characteristics and disease parameters of SLE and RA patients, respectively. Total CD34+ cells and levels of circulating EPCs, mEPCs and ECs are shown in Fig. 1B. No statistical differences between patients and HCs were detected in the CD34+ population; however, the SLE group tended to have an increased proportion of these cells (P = 0.062), being statistically significant when compared with RA (P = 0.002). Interestingly, a trend to reduce the CD34+ population was observed in RA related to disease progression, as patients at onset (n = 23) had significantly higher amounts than those recruited after diagnosis [674 (574)/10⁵ vs 313 (725)/10⁵, P = 0.020], despite the short duration of the disease [26 (23.75) months].

On the other hand, SLE patients exhibit higher EPC and mEPC absolute counts than HCs, whereas no significant
Next, we wanted to determine whether alterations in the size of the CD34\textsuperscript{+}, EPC or EC population was associated with a specific disease phenotype or treatment followed. Regarding CD34\textsuperscript{+} cells, no associations were detected with clinical or immunological features, but SLE patient users of anti-malarial treatment (alone or in combination) presented higher levels compared with non-users [802 (975) vs 353 (124), \(P = 0.018\)].

On the other hand, despite the increased EPC numbers detected in SLE, they were unrelated to disease characteristics or treatments. In early RA, a trend to increase this population was observed in patients with glucocorticoids \((n = 36, P = 0.068)\) and TNF-\(\alpha\) blockers \((n = 7, P = 0.08)\).

An interesting association was observed in the case of mEPCs in SLE because this population was higher in anti-SSA/B-positive patients as well as in those presenting malar rash when compared with their negative counterparts or with HCs (Fig. 2A). Moreover, we observed a trend towards increased CD34\textsuperscript{+} release but not EPC number, indicating that bone marrow precursors could be prematurely differentiated towards mEPCs. The analysis of early RA patients did not show any significant relationship with the amount of mEPCs.

Study of the EC population supports their relevance as a biomarker of endothelial damage (Fig. 2B). The amount of circulating ECs was higher in anti-SSA/B-positive SLE patients and there was a trend in early RA patients with anti-CCP antibodies \((P = 0.080)\). Looking at the effects of pharmacological treatments, both SLE and early RA untreated patients showed the highest EC levels (Fig. 2C), but no significant differences were detected between treatments. Of note, the EC number exhibited a positive correlation with disease activity in early RA (DAS-28: \(r = 0.259, P = 0.040\)) and a clear trend with anti-DNA titre in SLE \((r = 0.243, P = 0.057)\) patients. Other autoantibodies failed to display similar associations with the studied populations.
IFN-α levels were related to an increased mEPC population
To evaluate the possible relationship between EPC populations and cytokines involved in neovascularization and usually altered in autoinflammatory disorders, we quantified serum levels of VEGF-A165, IFN-α, TGF-β, IL-1β, IL-6, IL-8, IL-17 and GM-CSF in patients and controls at the time of EPC determination (Fig. 3). IFN-α was undetectable in most HCs, and IL-17 was only detected in 5 HC, 15 SLE and 35 early RA samples.

Serum levels of VEGF were decreased in SLE but not in early RA patients compared with HCs. Despite its vasculogenic potential, no association with EPC counts was found in any disease condition. Similarly, TGF-β levels, decreased in both diseases, did not associate with the number of EPCs. In contrast, serum levels of both molecules were negatively correlated with mEPCs in early RA patients (VEGF: \( r = -0.364, P = 0.002 \); TGF: \( r = -0.432, P < 0.001 \)), thus suggesting a protective role. Curiously, IL-17 levels, increased in early RA, were strongly

Fig. 2 Association between mEPC and EC populations and disease features.

(A) CD34+, mEPC and EPC levels in SLE patients presenting anti-SSA/B autoantibodies and malar rash. (B) EC levels in SLE and early RA patients with or without disease-specific autoantibodies. (C) Circulating ECs in SLE and early RA patients without treatment (n.t.) or after at least 3 months of treatment with glucocorticoids (GC), anti-malarials (AM), MTX (MT), immunosuppressive drugs (IS), LEF (LF) or TNF-α blockers (T-B) alone or in combination. Differences were evaluated by the Mann-Whitney U-test. *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.0001 \).
associated with VEGF in all individuals (HC: $r = 0.520$, $P = 0.005$; SLE: $r = 0.561$, $P < 0.001$; RA: $r = 0.472$, $P < 0.001$).

The most noticeable result was obtained by analysing IFN-α, a cytokine playing a central role in SLE and strongly up-regulated in these patients. However, early RA patients also showed increased levels of IFN-α, which were associated with disease activity (DAS-28: $r = 0.282$, $P = 0.028$).

Interestingly, as shown in Fig. 4A, serum levels of IFN-α correlated positively with CD34+ and mEPC populations in both diseases, but not with EPC, suggesting a specific role on mEPC premature differentiation. In fact, IFN-α increases in parallel with the mEPC/EPC ratio in early RA ($r = 0.234$, $P = 0.048$) and SLE ($r = 0.228$, $P = 0.061$), a balance indicative of vasculogenic EPC capability, suggesting the involvement of this cytokine in CD133 loss and EPC maturation. In addition, IFN-α levels were associated with EC number in both diseases, thus supporting the pathogenic role of this cytokine.

It was remarkable that in SLE patients, IFN-α increased at the same time as VEGF decreased ($r = -0.386$, $P = 0.004$), whereas no correlation was detected in HCs or early RA patients, in which VEGF was found at normal levels (Fig. 4B). On the contrary, IFN-α correlates positively with IL-6, a cytokine significantly augmented in both diseases. Furthermore, IL-6 correlated positively with CD34+ cells in all individuals (HC: $r = 0.533$, $P = 0.004$; SLE: $r = 0.263$, $P = 0.029$; early RA: $r = 0.235$, $P = 0.036$) and seemed to exhibit a similar ability to IFN-α in increasing the mEPC/EPC ratio in SLE ($r = 0.303$, $P = 0.012$). In the same way, IFN-α correlated directly with IL-1β in early RA and showed a trend in SLE patients. This cytokine, not significantly altered in patients, was also related to increased mEPC counts in both SLE ($r = 0.233$, $P = 0.05$) and early RA patients ($r = 0.301$, $P = 0.007$) and increased in parallel with the mEPC/EPC ratio in SLE ($r = 0.264$, $P = 0.03$).

Finally, IL-8 and GM-CSF levels were mutually correlated in SLE ($r = 0.313$, $P = 0.022$) and early RA ($r = 0.392$, $P = 0.001$) and significantly augmented in both diseases, but did not exhibit any significant relationship with EPC populations. Of note, both cytokines showed a strong positive correlation with IL-17 levels in SLE (GM-CSF: $r = 0.470$, $P < 0.001$; IL-8: $r = 0.559$, $P < 0.001$) and early RA (GM-CSF: $r = 0.655$, $P < 0.001$; IL-8: $r = 0.584$, $P < 0.001$), whereas IL-8 was directly correlated with VEGF in all groups (HC: $r = 0.741$, $P < 0.001$; SLE: $r = 0.483$, $P < 0.001$ and RA: $r = 0.562$, $P < 0.001$). All these results confirmed significant alterations in cytokine levels in both the diseases and suggest that IFN-α, and to a lesser extent IL-6 and IL-1β, are associated with increased EPC maturation and mEPC/EPC ratio, whereas VEGF and TGF-β seem to exert the opposite effect.

**Discussion**

Despite the current advances in EPC biology and its relevance in vascular homeostasis [5, 21], the most adequate
immunophenotyping technique remains unclear. In the present study, we included CD34 and CD133 as haematopoietic progenitor cell markers, and VEGFR2 as a specific endothelial-lineage marker, expressed by ECs in all differentiation stages [5]. We considered EPCs as triple-positive for these markers, in accordance with other authors [18, 25], whereas we contemplated the loss of CD133 as a sign of cell differentiation and maturation [5], giving rise to mEPCs. The use of these markers after pre-staining with a blocking reagent and a correct quantification by FACS, as performed in our study, are the most important recommendations of EUSTAR on EPC measurements [24].

This study showed an increased absolute number of circulating EPCs in patients with SLE, which was directly correlated with total CD34+ counts, suggesting an enhanced release of precursor cells from the bone marrow, probably, in some way, because of the secretion of soluble factors in response to systemic inflammation. In fact, serum levels of IL-6 and IFN-α were associated with an increase in the amount of circulating CD34+ precur-

sors. It has been reported that the elevated levels of nitric oxide (NO) present in the serum of autoimmune patients, which could be enhanced by IFN-α [26], can act on integrins, reducing their affinity to different ligands and leading to cell detachment from the bone marrow [27, 28]. Accordingly, reduced NO availability correlated with decreased numbers of EPCs in RA patients [15]. In addition, enhanced EPC release could be a repair response to the disseminated endothelial damage present in SLE patients [27].

Because different protocols have been used for EPC quantification in the studies performed with SLE patients, published results are heterogeneous and contradictory [21]. Only Grisar et al. [18] analysed CD34+VEGFR2+CD133+ cells, in a smaller SLE group (n = 31), showing no differences in EPC counts between HCs and SLE patients. In agreement with our results, other works reported high EPC levels, although they have not used the VEGFR2 marker [18, 29]. In contrast, some authors found reduced levels of CD34+VEGFR2+ [11, 13, 32] or CD133+VEGFR2+ cells [10]. Data about EPCs in RA seem to be less conflicting, as most authors reported normal or reduced levels [14, 15, 32]. Although our RA patients presented significantly less CD34+, EPCs and mEPCs than those with SLE, results did not reflect significant differences with controls. The relatively short disease duration of our RA cohort, as compared with other studies, could be an important source of discrepancies. In fact, we showed that all these populations tend to decrease after diagnosis. Of note, CD34+VEGFR2+CD133+ cells, considered as pre-EPC [33], were significantly reduced in RA patients
compared with both controls ($P = 0.049$) and SLE patients ($P = 0.017$). In addition to SLE, increased EPC counts have been detected in other rheumatic diseases [34–36]. It could seem paradoxical that in autoimmune diseases, characterized by a high risk of cardiovascular events, a cell population involved in maintaining vascular homeostasis was elevated rather than diminished. However, a defective functionality has been reported in EPCs from SLE patients, exhibiting accelerated senescence [13, 20], impaired migration or adhesive properties [18, 19] and reduced cluster formation [9]. The elevated IFN-$\gamma$ serum levels and/or the pro-inflammatory environment usually present in these patients could act on the EPC population, modulating their differentiation or impairing their functionality. Consequently, a deficient vascular repair could enhance progenitor cell release from bone marrow.

The quantification of CD133 EPCs (mEPCs) as a separate EPC population performed in this work allows us to achieve relevant results and supports the relevance of CD133 labelling for determining the true EPC population. Accordingly, a poor correlation has been reported between CD34$^+$VEGFR2$^+$CD133$^+$ and CD34$^+$VEGFR2$^-$ cells [37]. It has been proposed that mEPCs represent a mature subset of EPCs with little or no vasculogenic and/or repair capability [5]. In our work, this subset was significantly increased in SLE patients depending on their autoantibody status, as only anti-SSA and/or anti-SSB-positive patients presented increased levels of these cells. Furthermore, malar rash, associated with the presence of anti-SSA/B [38, 39], was the unique clinical feature associated with high mEPC counts. Accordingly, anti-SSA antibodies may isolate a subset of patients at higher risk of multiorgan vasculopathy [38] and were associated with increased intima-media thickness [31]. In line with this, a recent study by Kahlenberg et al. [40] showed that anti-SSA-positive SLE patients displayed an enhanced endothelial progenitor dysfunction due to high IL-18 levels and was associated with an inflammasome activation linked to IFN-$\gamma$. All these results support the use of these autoantibodies as biomarkers of defective vascular repair in SLE patients.

An interesting result of this work was the close relationship between IFN-$\gamma$ levels and mEPC counts, observed not only in SLE, but also in early RA patients, presenting a slight increase in IFN-$\gamma$ as well. Therefore our data suggest that IFN-$\gamma$ may play a role in bone marrow progenitor release and premature EPC differentiation towards the mEPC subpopulation and the consequent failure in endothelial repair. It has been reported that IFN-$\gamma$ could contribute to vascular damage and development of atherosclerosis in SLE by acting on EC apoptosis [20], platelet function [41], foam cell and monocyte recruitment [42] and, according to our results, the EPC population, impairing function, promoting apoptosis and leading to an ‘antiangiogenic signature’ [10, 11, 40, 43]. On the other hand, Thacker et al. [43], using a genomic approach, proposed that IFN-$\gamma$ could impair vascular repair on damaged endothelium by down-regulating VEGF expression, thus explaining the strong negative correlation observed in our SLE patients between IFN-$\gamma$ and VEGF levels.

Our results suggest that cytokines other than IFN-$\gamma$, for instance, IL-6 and IL-1$\beta$, could also promote EPC maturation, whereas TGF-$\beta$ and VEGF, probably involved in the maintenance and function of EPCs, may exert an opposite effect. Accordingly, Thacker et al. [43] showed that addition of IL-1$\beta$ to EPC cultures from SLE patients increased the number of mature ECs. However, our results suggest that the repressive effects of IFN-$\gamma$ on IL-1$\beta$ in EC cultures reported by Thacker et al. could be masked in vivo by the pro-inflammatory environment of autoimmune patients, specially the cytokine background, as a negative correlation between IL-1$\beta$ and IFN-$\gamma$ serum levels was not found. IL-6, linked to endothelial dysfunction in SLE [44] and RA [45], could disturb vascular repair by impairing EPC differentiation. On the other hand, despite the reported angiogenic role of IL-8 [46], GM-CSF [47] and IL-17A [48], we have not found any relationship between these cytokines and EPC populations. However, the strong positive correlation observed with VEGF leads us to hypothesize that they could be acting indirectly on EPC maintenance, up-regulating this growth factor.

Finally, EC results support their previously proposed use as a systemic endothelial damage biomarker. Actually these cells were increased in SLE patients with anti-SSA/B as well as in early RA patients presenting anti-CCP antibodies, in accordance with the reported association of these autoantibodies with subclinical atherosclerosis [37, 39, 49]. Thus the autoantibody profile and IFN-$\gamma$ levels, also associated with ECs, could serve in clinical practice to determine patients with elevated cardiovascular risk. It should be noted that, although ECs are usually identified by positive labelling for CD31 or CD146 markers, it has been shown that the majority of CD133$^+$ cells are CD146$^+$ [50]; thus we think that endothelial mature-specific labelling could be obviated, as the negative labelling for progenitor cells performed (CD34$^+$VEGFR2$^+$CD133$^-$) an adequate estimation of ECs detached from the endothelial wall.

In conclusion, this work shows that high IFN-$\gamma$ levels and/or the presence of anti-SSA/B antibodies may identify a group of SLE patients with increased mEPCs and, consequently, defective endothelial repair. The observed relationship between this population and IFN-$\gamma$ supports the use of therapeutic intervention blocking this cytokine in patients at high cardiovascular risk, thus proposing circulating IFN-$\gamma$ levels as a potential biomarker of endothelial repair status and responsiveness to anti-IFN-$\gamma$ therapy. Although the main limitation of this work is the lack of functional assays on patient EPCs to verify our hypothesis, this is the first study in which EPC, mEPC and EC populations were quantified and associated with disease features just as with cytokines involved in neovascularization or pathogenesis of SLE and RA, analysing >80 patients with each disease, being one of the largest EPC quantification studies in autoimmune diseases.
**Rheumatology key messages**

- Circulating mEPCs are increased in SLE patients with anti-SSA/B antibodies.
- IFN-α could promote EPC maturation in SLE and early RA patients.
- Disease-specific autoantibodies are associated with increased ECs in SLE and early RA.

**Acknowledgements**

The authors thank ALAS (Asociación Lúpicos de Asturias) for its continuous encouragement.

**Funding:** This work was supported by European Union FEDER funds, the Fondo de Investigación Sanitaria (FIS, PI080570) and the Fundación para el Fomento en Asturias de la Investigación Científica Aplicada y la Tecnología (FICYT, IB08-091). J.R.-C. is a recipient of a FPU grant from the Ministerio de Educación. C.P. was supported by a fellowship from FIS and B.dP. was supported by a fellowship from FICYT.

**Disclosure statement:** The authors have declared no conflicts of interest.

**References**


