Molecular Genetics and Metabolism Reports 12 (2017) 51–56

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism Reports

journal homepage: www.elsevier.com/locate/ymgmr

Short Communication

Adipose stem cells from obese patients show specific differences in the metabolic regulators vitamin D and Gas5

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ARTICLE INFO

Keywords:
Obesity
Adipose stem cells
Inflammation
Glucocorticoids
Gas5

ABSTRACT

Adipose tissue is a significant source of mesenchymal stem cells for regenerative therapies; however, caution should be taken as their environmental niche can affect their functional properties. We have previously demonstrated the negative impact of obesity on the function of adipose-derived stem cells (ASCs). Here we have evaluated other possible properties and targets that are altered by obesity such as the recently described long non-coding molecule Gas5, which is involved in glucocorticoid resistance. Using ASCs isolated from obese (oASCs) and control subjects (cASCs), we have analyzed additional metabolic and inflammatory conditions that could be related with their impaired therapeutic potential and consequently their possible usefulness in the clinic.

1. Introduction

Adipose tissue (AT) is a multifunctional and highly dynamic organ that, beyond its traditional role as a lipid storage site, is also a major endocrine organ [1] and an abundant source of stem cells [2]. AT secretes numerous factors, termed adipocytokines, which can originate from any cellular compartment (adipocytes, preadipocytes, immune cells, *etc.*,), and are major regulators of AT metabolism [3,4]. AT also serves as a novel source of adult stem cells, termed adipose stem cells (ASCs), which have great potential for therapeutic applications [5]. However, we and others have shown that the environmental niche in which ASCs reside has a profound impact on their functional properties [6–8].

Changes to AT mass are associated with endocrine and metabolic dysfunction and are linked to obesity [9] and a chronic inflammatory milieu [10], resulting in an altered cytokine profile that is often accompanied by insulin resistance and dyslipidemia [11]. Obese individuals present increased circulating levels of several inflammation markers [12,13] including Il-6, IL-8 and IL-1b, whose origin in AT is primarily the nonadipocyte component [14]. This overproduction of proinflammatory cytokines and the absence of anti-inflammatory cytokines contribute to the pathophysiology of obesity. AT-derived inflammatory adipocytokines are also secreted into systemic circulation where they exert profound effects on the hypothalmic-pituritary-adrenal axis by upregulating the expression of corticotropin-releasing hormone (CRH) and increasing the production of glucocorticoids (GCs) [15].

GCs have well-established effects on the metabolic regulation of AT homeostasis [16,17], and are required for glucose and fatty acid metabolism [11,18]. They also play an important role in inflammation associated with obesity since the glucocorticoid biosynthetic pathway enzyme, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), is upregulated selectively in AT in obesity. While circulating GC levels are normal in obesity [19], 11β-HSD1 converts inactive cortisone back to active cortisol in AT [20], and increasing local cortisol levels are implicated in the pathogenesis of the metabolic syndrome [21]. The action of GCs on target cells is thought to be mediated by the glucocorticoid receptor (GR), whose binding by GC agonists triggers its translocation into the nucleus [22] where it dimerizes and binds directly to glucocorticoid-responsive elements (GREs) to stimulate the expression and regulation of different genes. GR can be regulated by other factors, such as the recently discovered Gas5 component.

Long noncoding RNAs (lncRNAs) are a novel class of functional RNAs that control gene regulation [23]. Many of them are associated with differentiation and homeostasis of metabolic tissues, such as the growth arrest-specific 5 (Gas5) lncRNA, which is induced under cellular stress or starvation conditions [24]. This lncRNA is a transcript of Gas5, a non-protein coding gene [25], which acts as a repressor of the GR through binding of a decoy GRE to ligand-activated GR [26], thereby suppressing the transcriptional activity of GC-responsive genes. Along

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http://dx.doi.org/10.1016/j.ymgmr.2017.05.008
Received 14 February 2017; Accepted 22 May 2017
Available online 27 May 2017
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this line, vitamin D deficiency is known to be related to obesity [27], and vitamin D can influence adipocytokine production and inflammation in AT [28].

Several independent lines of evidence demonstrate the dysregulated function of ASCs isolated from AT of obese patients [8,29]. We have also demonstrated impaired differentiation [30], migration [31] and metabolic properties [6,32] of ASCs isolated from obese individuals. Here, we examined additional metabolic factors in this stem cell population that may have utility for their functional characterization.

2. Materials and methods

2.1. Reagents

Dulbeco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) was purchased from Sigma (St. Louis MO). Penicillin, streptomycin, L-glutamine and Hepes was from Lonza (Basel, Switzerland). Kits for molecular studies were purchased from Applied Biosystems, Life Technologies (Paisley, UK). Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich.

2.2. Isolation, culture and adipogenesis of ASCs

Human adipose-derived stem cells (ASCs) from non-obese and obese (body mass index < 22 kg/mg^2 and > 30 kg/m^2, respectively) patients were isolated and characterized as described [30,33]. Subcutaneous adipose tissue was obtained from patients after bariatric surgery (obese patients) or normal surgery (non-obese patients) at the Hospital Universitario de la Princesa, Madrid (females aged 35–45 years; 5 obese and 5 non-obese). Sample collection conformed to the principles of the Declaration of Helsinki and the NIH Belmont Report. The ethics committee of the Centro Nacional de Investigaciones Cardiovasculares approved the use of human samples. Cells were isolated, sorted and expanded as described [34], and cultured in DMEM supplemented with 10% FBS at 37 °C in a humidified 5% CO_2/95% air atmosphere. Adipogenic differentiation was performed using 2×10^4 cells plated into 24-well plates in DMEM/FBS. After 24 h, the medium was exchanged for adipocyte growth medium (STEMPRO Adipogenesis Differentiation Kit) supplemented with 1 μM dexamethasone and cells were cultured for up to 7 d. Triglyceride accumulation was visualized by staining paraformaldehyde (PFA)-fixed cells with Oil Red O, and images were taken with an IX71 inverted microscope.

2.3. Flow cytometry analysis

ASCs were stained with the following antibodies against surface markers: CD45-V450, CD34-PE, CD44-Alexa488, Sca1-PeCy7 and ckit-APC, all at 1:100 (BD Biosciences, San Jose, CA, USA). After incubation for 25 min at 4 °C, stained cells were analyzed by flow cytometry on an LSRFortessa flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed with BD FACSDiva Software.

2.4. Quantitative real-time polymerase chain reaction

Total RNA was extracted from ASCs with TRI Reagent and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the primers shown in Table 1. Each cDNA sample was amplified in triplicate using a SYBR Green PCR Master Mix. PCR mixes were loaded in an AB 7900 Fast Real-Time PCR System and quantified using SDS 2.0 software.

2.5. Vitamin D analysis

Cells and culture medium were separated by centrifugation at 5725 × *g* for 5 min at 4 °C. Cell pellets containing equal numbers of each cell line were subjected to three freeze-thaw cycles as described [6] to obtain cellular extracts. Culture medium and cellular extracts were prepared for liquid chromatography-mass spectrometry (LC-MS) to quantify total vitamin D derivatives as described [32]. Samples for each technique were prepared with replicates as quality control. Values are expressed as the “percentage of change” between groups.

Table 1
Primer sequences for qRT-PCR.

	Primer forward sequence (5′-3′)	Primer reverse sequence (5′-3′)
β-Actin	*CACGATGGAGGGGCCGGACTCAT*	*TAAAGACCTCTATGCCAACACAG*
Gas5	*AGCTGGAAGTTGAAATGG*	*CAAGCCGACTCTCCATACC*
IL-1	*GGGCCTCAAGGAAAAGAATC*	*TTCTGCTTGAGAGGTGCTGA*
IL-6	*TACCCCCAGGAGAAGATTCC*	*TTTTCTGCCAGTGCCTCTTT*
IL-8	*GTGCAGTTTTGCCAAGGAGT*	*CTCTGCACCCAGTTTTCCTT*
IL-10	*TGCCTTCAGCAGAGTGAAGA*	*GGTCTTGGTTCTCAGCTTGG*
MCP-1	*CCCCAGTCACCTGCTGTTAT*	*TGGAATCCTGAACCCACTTC*
Icam-1	*GGCTGGAGCTGTTTGAGAAC*	*ACTGTGGGGTTCAACCTCTG*

2.6. Data analysis

Statistical analysis was performed using the GraphPad Prism software package (GraphPad, San Diego, CA). Comparison between groups was performed by one-way or two-way analysis of variance as required by the assay. Values were expressed as mean ± SEM or mean ± SD, and data was considered significantly different at $p < 0.05$. Analysis of LC-MS data was carried out by univariate (UVA) and multivariate (MVA) analysis using MATLAB R2015 software (Mathworks, Inc., Natick, USA) and SIMCA P+ 12.0.1 software (Umetrics, Umea, Sweden), respectively. For UVA, the non-parametric Mann-Whitney *U* test with Benjamini-Hochberg *post hoc* correction (level $q < 0.05$) was applied, and for MVA, log-transformed and Pareto-scaled data, or just Pareto-scaled data, were used to create multivariate models.

3. Results

3.1. Adipose stem cell isolation and characterization

ASCs derived from subcutaneous depots from non-obese (cASC) and obese (oASC) donors were isolated using an explant method [30,33]. Cells were characterized by flow cytometry and were positive for the surface markers CD34, CD44, Sca1 and ckit and negative for CD45 (Fig. 1A). Comparable ASC populations were obtained from non-obese and obese subjects as characterized by specific stem cell markers [34].

3.2. Gas5 expression is enhanced during adipogenesis

We examined Gas5 expression in ASCs grown under normal conditions and after adipocyte differentiation. No differences in the levels of Gas5 mRNA were found between cASC and oASC under basal conditions (Fig. 1B). We induced adipocyte differentiation by culturing cells in STEMPRO medium with dexamethasome. After 7 days, differentiation was confirmed by the presence of accumulated triglycerides evaluated with Oil red O (Fig. 1C), and Gas5 expression was measured. Whereas adipogenesis increased Gas5 expression both in cASC and oASC 4–5-fold, the increase was significantly lower in oASC (Fig. 1B).

3.3. Inflammatory and metabolic conditions

Obesity is associated with insufficiency of vitamin D [35], which acts an antioxidant and has pleiotropic effects including an anti-inflammatory action. Having shown that oASCs have an altered metabolic profile [6,32], we assessed cellular and secreted vitamin D levels in the two ASC groups. The level of cellular vitamin D was significantly lower (60%) in oASC than in cASC, and this was paralleled

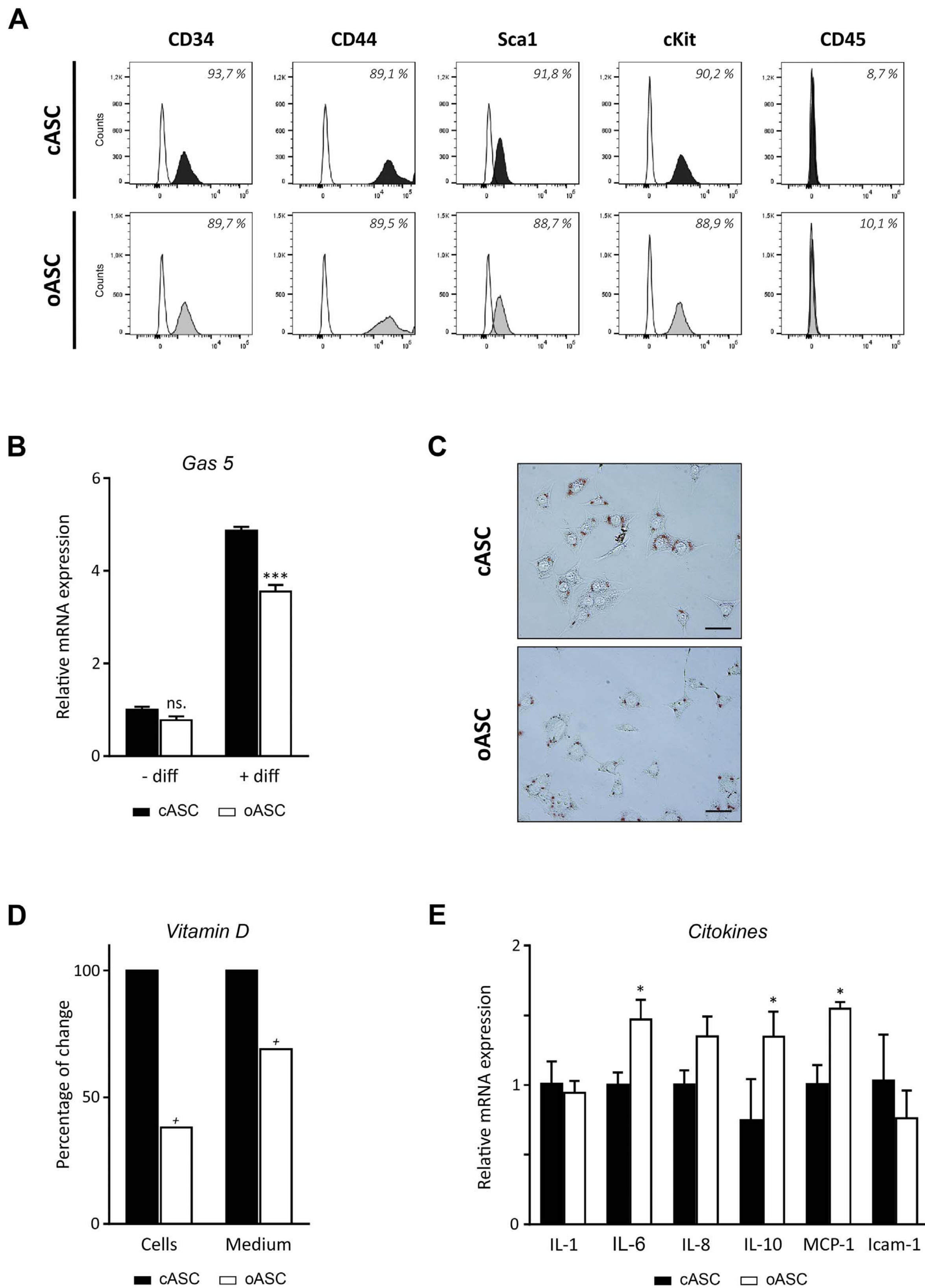

Fig. 1. A. Analysis of cell surface markers by flow cytometry in adipose stem cells from control (cASCs) and obese (oASCs) subjects. Positively stained cells are expressed as a percentage in the plot. Histograms show isotype-matched controls and fluorescence intensity of each cell surface marker and are representative of three independent experiments with similar results. B. Representative image of Oil Red O staining in cASCs and oASCs differentiated for 7 days into adipocytes. Scale bar, 30 μm. C. Gas5 gene expression by cASCs and oASCs under basal conditions (− diff) and at day 7 differentiation (+ diff). ***$p < 0.001$ (Anova). D. Vitamin D quantification. Graph represents the percentage of change within the cells and in the medium. + $p < 0.05$. E. Gene expression profile of different cytokines in cASCs and oASCs. *$p < 0.05$.

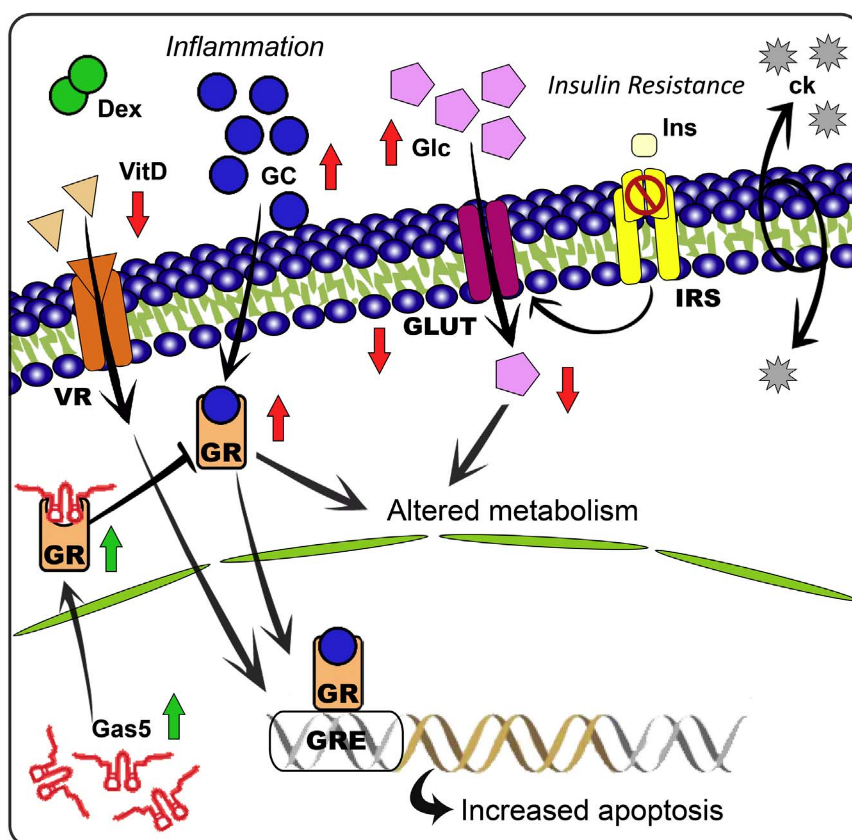

Fig. 2. Adipose tissue inflammation in obese conditions induces alterations in adipose stem cells. Cytokines are increased in obese conditions, triggering an enhanced glucocorticoid production as an anti-inflammatory response. Dexamethasone and adipogenic components promote adipogenic differentiation and induce an altered response in different metabolic pathways. Dex, Dexamethasone; Gas5, Growth arrest-specific 5; GC, Glucocorticoids; GR, Glucocorticoid receptor; GRE Glucocorticoid response element; Glc, Glucose; GLUT, Glucose transporter; Ins, Insulin; IRS, Insulin receptor substrate; VitD, Vitamin D.

by a significant decrease in secreted levels (30%) in oASC (Fig. 1D). This result is line with the findings that individuals with obesity present a reduction in vitamin D.

An inverse relationship between vitamin D levels and inflammation markers has been described in obese patients [36]. To determine whether obesity and vitamin D deficiency resulted in increased cytokine production in ASCs, we measured the expression pattern of a panel of cytokines and Icam-1. Expression of IL-6, IL-8, IL-10 and MCP-1 was higher in oASC than in cASC, whereas no differences in expression were detected for IL-1 and Icam-1. These results suggest an increased inflammatory response in oASC due to obesity and vitamin D reduction.

4. Discussion

While ASCs hold great promise for regenerative medicine applications [37], obesity leads to a reduction in the ASC pool [38,39] and we have previously shown that AT from obese donors has a negative effect on the ASC population, impacting their stemness and metabolic properties [6,30,31].

We show here that whereas oASC and cASC have similar Gas5 expression levels under basal conditions, expression is lower in oASC than cASC during adipogenic differentiation. Gas5 levels are increased during adipogenic differentiation due to the presence of dexamethasone in the medium, but this synthetic glucocorticoid presents a reduced action on oASCs.

Some studies have suggested an association between obesity and vitamin D deficiency, as obese individuals tend to have low vitamin D levels [35]. We found a decrease in vitamin D levels in oASC, both in the medium and in cells, confirming this association. Additionally, obesity results in a blood flow imbalance leading to inflammation, and macrophage infiltration [40], and it has been demonstrated that vitamin D metabolites influence adipcytokine production and the inflammatory response in AT [28]. Accordingly, we detected a cytokine profile imbalance between oASC and cASCs. Thus, obesity affects vitamin D levels and promotes an altered cytokine profile that is inflammatory. It remains to be tested whether vitamin D decreases the release of the main pro-inflammatory molecules in AT.

Our findings allow us to propose the following model (Fig. 2). Obesity provokes a marked inflammatory process within AT, principally in macrophages [41]. This inflammation is associated with changes in the microenvironment (red arrow Fig. 2) characterized by abnormal adipocytokine production and proinflammatory signaling pathway activation [9]. In parallel, GC levels increase due to enhanced levels of 11βHSD1 [11], which results in an altered metabolism due to GC-regulated pathways such as glucose [42] and lipid [43] metabolism. Furthermore, it has been reported that GCs act synergistically with insulin, and can improve or impair their action [11]. GCs are regulated by the noncoding RNA Gas5, which is an associated repressor of the GR [44]. During dexamethasome-induced lipogenesis, Gas5 levels increase to repress GC action [45], but Gas5 levels in oASC are lower than in cASCs (Fig. 1C), hindering lipid metabolism. We have previously shown that ASCs isolated from obese subjects have significantly enhanced apoptosis and also a reduced proliferative capacity [6]. In this regard, GCs regulate apoptosis and exert significant anti-proliferative effects [46], and GC dysregulation supports our previous findings in relation to apoptosis and proliferation.

Several *in vitro* studies have demonstrated that vitamin D inhibits chronic inflammation in AT [47]; however, vitamin D levels are decreased in obese subjects [35]. The present findings are in accordance

with this and we hypothesize that low amounts of vitamin D may increase the metabolic risk and contribute to the inflammatory process. Furthermore, vitamin D enhances GC responsiveness by increasing its anti-inflammatory activity [48], and we found a misbalance in those components in oASCs. Concomitant with this, we detected increased cytokine expression, and it has been described that chronic activation of pro-inflammatory pathways within AT may impair metabolic control [49]. Thus, the increased inflammatory state and GC release activate anti-inflammatory pathways and GCs down-regulate the expression of several inflammatory genes, including those encoding cytokines [50].

ASCs have been used in several studies and preclinical data indicate that ASCs present high safety and efficacy, supporting their use in cell-based regenerative medicine. The data in the present study confirm and extend our previous findings showing that ASCs present different biological properties due to the obese microenvironmental niche. Obesity-derived signals, inflammatory and others, play a key role in ASC impairment and our present findings can help us to understand the mechanisms responsible for these metabolic alterations.

Funding

This study was supported by a grant from the Spanish Ministry of Economy and Competitiveness (SAF 2015-67911) to BGG. BdL is supported by FPU fellowship from the Spanish Ministry.

Acknowledgements

We would like to thank Dr. Kenneth McCreath for critically reviewing the manuscript.

L.M.P. and B.dL. researched data. All authors contributed to discussion and reviewed the manuscript. L.M.P. and B.G.G. wrote the manuscript and discussed data.

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