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Serum leptin levels correlate negatively with the capacity of vitamin D to modulate the *in vitro* cytokines production by CD4$^+$ T cells in asthmatic patients

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ABSTRACT

Both obesity and low vitamin D levels have been associated with allergic asthma (AA) severity. In the present study, severity of AA was associated with obesity but to the *in vitro* IgE production. In those patients, higher levels of IL-5, IL-6 and IL-17 were quantified in CD4$^+$ T-cell cultures as compared with patients with mild and moderate AA. In addition, the lowest IL-10 levels were detected in the cell cultures from patients with a worse prognosis. Interestingly, the occurrence of AA elevates the plasma levels of leptin, and this adipokine was positively correlated with the release of IL-5, IL-6 and IL-17, but inversely correlated with IL-10 production, by CD4$^+$ T-cells from patients. In AA-derived CD4$^+$ T-cell cultures, 1,25(OH)2D3 was less efficient at inhibiting IL-5, IL-6 and IL-17 production, and up regulating IL-10 release, as those from healthy subjects. Interestingly, the *in vitro* immunomodulatory effects of vitamin D were inversely correlated with serum leptin levels. In summary, our findings suggested that obesity, probably due to the overproduction of leptin, negatively impacts AA as it favors imbalance between Th2/Th17 and regulatory phenotypes. The deleterious effects of leptin may also be due to its ability to counter-regulate the immunosuppressive effects of vitamin D.

1. Intriduction

Allergic asthma (AA) is an immune-mediated reaction of the airways to inhaled allergens characterized by recurrent episodes of breathing difficulty that affects approximately 300 million people worldwide [1]. The disease may significantly impair the patient's quality of life and lead to very high healthcare costs, thus representing a major public health problem worldwide [1,2]. The acute symptoms of asthma include wheezing, coughing and shortness of breath that can lead to airway tissue remodeling that is characterized by increased smooth muscle mass, fibrosis, and mucus production. This airway hyperreactivity (AHR) leads to airflow obstruction that can be life threatening.

The prevalence of AA has been increasing over recent decades, and it is believed that risk factors associated with modern-life style, such as obesity, may contribute to the increase of allergic diseases in genetically predisposed and epigenetically regulated individuals [3].

Obesity increases asthma severity and compromises pharmacological treatment [4–7]. Given that obesity is associated with pro-inflammatory status [8], it is possible that cytokines and adipokines modulate the behavior of immune cells involved in the immune-pathogenesis of AA.

Classical AA is associated with allergen-specific Th2 cells that produce high IL-4, IL-5 and IL-13 levels [9,10]. The differentiation of this phenotype depends on dendritic cell (DC) subsets able to present processed peptides from allergens associated with MHC class II molecules

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in association with IL-4 release [11,12]. Furthermore, epithelial cells (EC) from asthmatic patients, by releasing high levels of IL-25 [13], IL-33 [14] and thymic stromal lymphopoietin protein (TSLP) [15], favor the development of a pro-allergic DC phenotype [16,17]. These EC-derived cytokines also potentiate IL-5 and IL-13 production by local Group 2 innate lymphoid cells (ILC2) [18–20]. A hallmark of this response is the production of allergen-specific IgE by B cells [21]. The clinical symptoms of AA are particularly a result of biological actions of pre-formed and newly synthetized pro-inflammatory mediators released by IgE-sensitized mast cells and mainly eosinophils after cross-linking of these antibodies by allergens. In the context of AA, sulphidopeptide leukotrienes (LTC4, LTD4, and LTE4) and platelet-activating factor (PAF) [22,23] play a pivotal role in the pathogenesis of acute attacks due to their ability to provoke local vasodilatation, edema formation, local neurogenic stimulation, smooth muscle contraction and mucus hypersecretion.

The control of acute asthma attacks involves the use of β2 agonists (bronchodilators) and inhaled corticoids (ICs) [24,25]. Unfortunately, in a proportion of patients, symptoms are not adequately despite high doses of these drugs [26], and Recent studies have suggested an involvement of another $CD4^+$ T cell phenotype in those patients, the Th17 cells.

In humans, IL-17-producing $CD4^+$ T cell differentiation can be induced by IL-6 and IL-23 produced by DCs [27]. An increased level of both IL-17 and IL-23 in the serum and lungs of patients with severe asthma were reported [28]. The immunological pattern of this type of asthma involves neutrophilic airway inflammation associated with intense local damage and resistance to ICs [29,30]. Moreover, a mixed pattern of Th2 and Th17 cells appears to co-exist in some asthmatic patients, which also tends to be refractory to ICs therapy than patients with the classical Th2 pattern [31,32]. Corticoid resistance mechanisms are not well understood but they probably involve the ability of IL-17 to up-regulate GRβ, while attenuating the expression of the functional corticoid receptor, GRα isoform [33].

Obesity complicates eosinophilic and neutrophilic AA. Obese asthmatic patients do not respond as well to ICSs and adrenergic β2 agonists [34–36], a phenomenon probably related to increased production of inflammatory cytokines [34]. Obesity is characterized by an increased pro-inflammatory M1 to anti-inflammatory M2 macrophage ratio in adipose tissue [37]. While M2 is linked to wound healing by attenuating inflammation through release of IL-10 and TGF-β, M1 cells produce high levels of pro-inflammatory cytokines (IL-12, IL-6, TNF-α, IL-23) and free radicals derived from oxygen (ROS) and nitrogen (NO) [38,39]. In addition to its local pro-inflammatory pattern, adipose tissue can modulate systemic immune response through release of adipokines, such as leptin and adiponectin [8]. While adiponectin favors expansion of M2 and Tregs cells, leptin plays a pro-inflammatory role, damaging regulatory T cell function, which compromises the production of anti-inflammatory cytokines, such as IL-10 [40]. An imbalance in the expression of receptors for both adipokines in T cells of obese individuals, with up regulation of leptin receptor, being reported [41,42].

By inducing the production of TNF-α and IL-6, obesity-associated levels of leptin favor activation of effector T cells, mainly Th17 phenotype, and damage Treg function, which reduces the production of anti-inflammatory cytokines such as IL-10 [40,43–48]. Interestingly, high leptin levels were inversely correlated with lung function in asthmatic patients [49].

In addition to promoting high leptin levels, lower circulating vitamin D levels observed in obese patients [50] could also negatively impacts the immune-pathogenesis of asthma. In a study by Santos et al. [50], the prevalence of vitamin D deficiency was 35% higher in obese individuals compared to the eutrophic group. More severe asthma symptoms in children have been associated with poor maternal intake of vitamin D during fetal development [51]. There is a growing literature on the association between vitamin D deficiency and the risk of exacerbated asthma [52,53] and corticosteroid resistance [54–59] in patients.

Apart from its regulatory role in calcium homeostasis, vitamin D appears to play a protective role in the context of inflammatory disorders, which is probably due to its ability to modulate many aspects of the immune response. In $CD4^+$ T cells from healthy individuals, the active form of 1,25 dihydroxyvitamin D [1,25(OH)2D3] has been shown to elevate liberation of IL-10, and reduce production of IFN-γ and IL-17 [60–62]. The effect of 1,25(OH)2D on T cells may be indirect, by down-regulating the immunogenic function of antigen presenting cells, such as DCs. Indeed, 1,25(OH)2D not only reduces the expression of CD80, but also diminishes the production of pro-inflammatory cytokines by LPS-maturated DCs [63]. Whether the levels of circulating leptin correlates with the ability of vitamin D to regulate the cytokine profile of T cells in asthmatic patients is still unclear. Therefore, the objective of the present study was to investigate the immunomodulatory affects of 1,25(OH)2D3 on the *in vitro* cytokine profile of T cells from lean and overweight/obese asthmatic patients are related to the *in vivo* leptin levels.

2. Methods

2.1. Subjects

Sixty patients with AA (40 women and 20 men) were recruited from March 2017 to November 2018 from Federal University of the State of Hospital/UNIRIO (Rio de Janeiro, Brazil). Pulmonary functions were assessed by spirometry according to American Thoracic Society standards [64]. Asthma severity was evaluated on the basis of the Global Initiative for Asthma criteria [65]. Asthmatic subjects were subdivided into 3 groups: mild ($n = 20$), moderate ($n = 20$) and severe ($n = 20$) asthma. Patients were allowed to receive treatment with ICs, but not systemic steroids for 2 months prior to the study. All patients had a positive skin prick test, defined as a > 5-mm diameter skin wheal response to at least 1 of 8 common allergens (*Dermatophagoides pteronyssinus*, *D. farinae*, *Alternaria*, mixed grass pollen, mixed tree pollen, dog and cut hairs and cockroach). The occurrence of infectious or other autoimmune diseases were excluded by clinical and serological tests. Sixty healthy subject, matched by age, gender and body mass index (BMI) were recruited for the control group. BMI is calculated from the mass (weight in kg) and height (in meters) of an individual taking in account the formula ($BMI = kg/m^2$). Then the subjects were stratified as lean (BMI from 18.5 to 24.9), overweight (BMI from 25 to 29.9) and obese class I (BMI from 30 to 35). In the present study, all subjects included were nonsmokers, with no history of upper or lower airway infectious diseases 4 months prior to recruitment in the study, no chronic heart or pulmonary diseases; and did not receive oral or intravenous steroids, theophylline, long-acting β2-agonists, leukotriene antagonists or antihistamines 1 month prior to the study. The Ethics Committee for Research on Human Subjects at the Federal University of the State of Rio de Janeiro (UNIRIO) approved the study and blood was collected only after written informed consent was obtained from each individual. (CAAE 44951215.6.0000.5258).

2.2. Cell cultures and stimuli

Peripheral blood mononuclear cells (PBMCs) from 30 mL of blood were separated by centrifugation on Ficoll–Hypaque gradients. The viable cells, measured by trypan blue exclusion, were adjusted to a concentration of 1×10^6 cells/mL and cultured in 24-well flat bottom microtiter plates in 1 mL RPMI 1640 adding 2 mM L-glutamine, 10% fetal calf serum, 20 U/mL penicillin, 20 μg/mL streptomycin, and 20 mM HEPES buffer (GIBCO, Carlsbad, California, USA). In some experiments, enriched $CD4^+$ T cells and B cells were obtained *via* negative selection using magnetic columns according to manufacturer's instructions (EasySep™, StemCell Technology, Canada). Briefly, 50 μL of the

isolation cocktail was added to a cell suspension (1×10^7 cells/1 mL) in a 14 mL tube. After rapidly mixing, the suspension was incubated for 10 min at room temperature. Then, already homogenized RapidSphere suspensions were added to the cell suspension at 100 µL for $CD4^+$ T cells and 150 µL for $CD19^+$ cells (B cells). After rapidly mixing, the cell suspension was incubated at room temperature for 5 min. Finally, 4 mL of HBSS solution was added to the cell suspension and, after pipetting, the tube was then placed on the magnet for 5 min and supernatants were recovered. The purity of $CD4^+$ T cells and B cells was > 98%, as measured by flow cytometry (data not shown). Both PBMC (1×10^6/mL) and enriched $CD4^+$ T cell (1×10^5) cultures were maintained for 72 h in the absence (medium alone) or presence of anti-CD3/anti-CD28 beads (10 µl/mL) at 37 °C and 5% CO_2. To evaluate the *in vitro* production of IgE in co-culture system, $CD4^+$ T cells (1×10^4/500 µL) and B cells (1×10^4/500 µL) were stimulated with 1 µg/mL of Staphylococcal enterotoxin B (SEB) from *Staphylococcus aureus* (Sigma-Aldrich Co) for 6 days. The *in vitro* effect of vitamin D was evaluated after adding 10 or 20 ng/mL of 1,25 dihydroxyvitamin D3 [1,25(OH) 2D] at the beginning of cell cultures. The dose of vitamin D used here was based on the ability of different 1,25 (OH)2D concentrations (1, 5, 10 and 20 ng/mL) to modulate the release of IL-6 by polyclonally-activated T cell cultures from healthy subjects (data not shown). Notably, the 1,25(OH)2D did not affect cell survival, determined by both trypan blue exclusion and Propidium iodide. The cell cultures were maintained at 37 °C in a humidified 5% CO_2 incubator.

2.3. Quantification of cytokines

The quantification of cytokines secreted by polyclonally-activated T cells was performed in by using OptEIA ELISA kits (BD, Pharmigen, San Diego, CA), according to manufacturer's instructions. Briefly, each assay was performed using pairs of mAbs directed to human IL-10, IL-6, IFN-γ, IL-4, IL-5 and IL-17. The reaction was revealed with streptavidin horseradish peroxidase, using 3,3′,5,5′-tetramethyl-benzidine (TMB) as substrate. Recombinant human cytokines, at concentrations ranging from 3.5 to 500 pg/mL, were used to construct standard curves.

2.4. Plasmatic vitamin D measurement

Plasma 25(OH) vitamin D concentration was measured using a commercially available ELISA kit (Immunodiagnostik, Bensheim, Germany) as per the manufacturer's instructions. The 25(OH)D3 levels are normally measured since 1,25(OH)2D is more stable (44). According to the classification proposed by Holick & Chen (2008), normal vitamin D levels were defined as being higher than 30 ng/mL.

2.5. Leptin and IgE quantification

Circulating leptin levels were measured from the harvested plasma and quantified by ELISA using Leptin kit (Human) following manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY). The concentration of IgE secreted by B cells in the co-culture system was determined by human IgE ELISA kit (ab108650) (Abcam co). Briefly, 100 µL of the samples from plasma (leptin) or supernatants of co-cultures (IgE) were added to each well containing anti-leptin or anti-IgE primary antibody, respectively. Then, the wells were incubated for 1 h, washed and treated with 100 µL of the biotinylated secondary IgG antibody for anti-Leptin or for anti-IgE. Finally, 100 µL of the streptavidin-horseradish peroxidase conjugate enzyme was added to the wells and then the TMB substrate (3,3,5,5′-tetramethylbenzidine). Plates were read at 450 nm in ELISA reader (Dynex Technologies, USA). Lyophilized leptin ranging from 31.3–2000 pg/mL was used to construct the standard curve. Regarding IgE, the standard curve was constructed from 10 to 800 IU/mL of recombinant human IgE.

2.6. Statistical analysi

Statistical analysis was performed using Prism 5.0 software (GraphPad Software). All immunological evaluations were performed in triplicate or quadruplicate in each individual and the intra-assay variability ranged from 8.7 to 15.1% (median value of 10.1%) as calculated by the software. To compare > 2 groups, we used one-way ANOVA followed by the Turkey test for data with Gaussian. The non-parametric Mann-Whitney *U* test and the Student's *t*-test were applied to determine whether the two groups were statistically different for nonparametric and parametric variables, respectively. Correlations between variables were investigated using Pearson's correlation. Significance in all experiments was defined as $p < .05$.

3. Results

3.1. Characteristics of AA patients and impact of obesity and severity of disease on the production of cytokines by T cells

For our study, 60 patients (40 female and 20 male) with mild ($n = 20$), moderate ($n = 20$) and severe ($n = 20$) allergic asthma (AA) were recruited and stratified by body mass index (BMI). It is worth noting that patients with severe asthma were overweight ($n = 4$) or obese class I ($n = 16$). In patients with moderate AA, 6 were lean, 10 overweight and 4 obese (class I). Among patients suffering from mild AA, 12 were lean, 6 overweight and 2 had obesity class I. Insufficient mean values of circulating 25(OH)2D3, defined as values < 30 ng/mL, were detected in healthy subjects and AA patients, without any statistical difference (Table 1).

The first immune assay performed was the analysis of the cytokine profile of activated T cells in PBMC cultures in response to mitogen. As can be observed in Fig. 1A, significantly higher levels of IL-6, IL-17, IL-4 and IL-5 were measured in AA patients when compared with the control group. In contrast, IFN-γ production was higher by activated T cells from healthy subjects. No significant difference was observed regarding IL-10 secretion in both experimental groups (Fig. 1A). Notably, no cytokine was detectable in unstimulated PBMC cultures (medium alone) from any subject. When cytokine production was stratified by BMI, no statistical difference was observed concerning almost cytokines secreted in the control cultures, except IL-6 that was produced in higher

Table 1
Characteristics of subjects.

	Control[a]	Asthma[b]
No of subjects (n)	60	60
Gender, female/male (n)	40/20	40/20
Age [(years), mean ± SD]	39.1 ± 17.1	38.9 ± 16.3
Plasmatic vitamin D [mean (range)][c]	25.9 (8.1–117)	22.1 (5.3–67)
BMI (n)[d]		
Lean	18	18
Mild	ND	12
Moderate	ND	6
Severe	ND	0
Overweight	20	20
Mild	ND	6
Moderate	ND	10
Severe	ND	4
Obese class I	22	22
Mild	ND	2
Moderate	ND	4
Severe	ND	16

[a]Healthy individuals and [b]patients with mild ($n = 20$), moderate (n = 20) and severe (n = 20) allergic asthma. [c]Plasmatic 25(OH) vitamin D concentrations in ng/L were measured using commercially available ELISA kit. [d]Body mass index: is a value derived from the mass (weight in kg) and height (in meters) of an individual (lean: 18.5–24.9, overweight: 25–29.9 and obese class I: 30–35). ND = no determined.

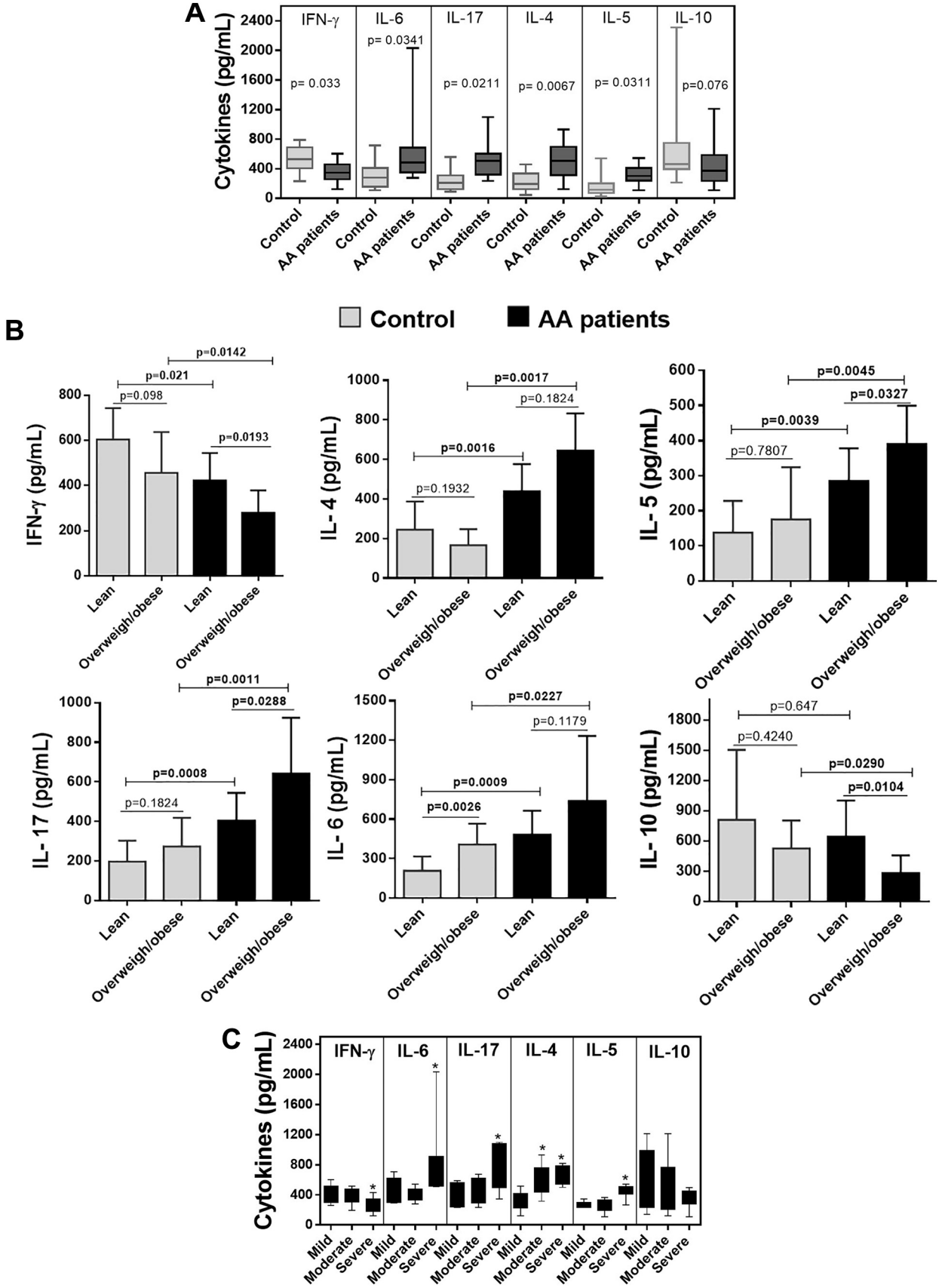


Fig. 1. Cytokine production by polyclonally-activated T cells from asthmatic patients as a function of obesity and clinical status. In (**A**), the *in vitro* production of cytokine by PBMC (1×10^6/mL) from healthy ($n = 60$) and asthmatic patients ($n = 60$) was evaluated by ELISA after activating T cells with anti-CD3/anti-CD28 beads (10 μL/mL) for 3 days. In (**B**) and (**C**), cytokine levels were stratified by BMI and severity of allergic asthma (AA), respectively. In (**A**) and (**B**), the mean values were compared and the *p* values are shown in the figure, In (**C**), (*) indicates $p < .05$.

levels by T cells from overweight subjects (Fig. 1B). In AA patients, the secretion of IFN-γ was lower, especially among overweight/obese subjects. By contrast, higher IL-4, IL-5, IL-6 and IL-17 levels were detected in the supernatants of activated T cell cultures from AA patients, mainly among overweight/obese ones (Fig. 1B). Elevated BMI reduces the ability of polyclonally-activated T cells from patients to produce IL-10 (Fig. 1B). Concerning clinical status, elevated levels of IL-5, IL-6 and IL-17 were observed in cell cultures from patients with severe asthma (Fig. 1C). Moreover, IL-4 levels were significantly higher in those with moderate and severe AA (Fig. 1C). IFN-γ release was compromised in cell cultures from patients suffering from severe AA (Fig. 1C). No difference was observed between disease severity and IL-10 production (Fig. 1C).

3.2. The role of 1,25 (OH)2D3 in modulating cytokine production by T cells from AA patients

The active form of vitamin D, 1,25(OH)2D3, is known to favor the production of IL-10 and reduce pro-inflammatory cytokines by human T cells (60–63). As compared to the control group, the active form of vitamin D was less efficient at reducing the production of cytokines related to the Th1 (IFN-γ), Th2 (IL-4 and IL-5) and Th17 (IL-6 and IL-17) phenotypes in AA patients. Moreover, 1,25(OH)2D3 was less potent at increasing IL-10 secretion by AA-derived activated T cells (Fig. 2A). Among the patients, the effect of 1,25(OH)2D3 for modulating cytokine production was impaired in overweight/obese patients, mainly those suffering from severe asthma (Fig. 2B).

3.3. Relationship between systemic leptin levels and in vitro production of cytokines by T cells of AA patients

Although both experimental groups were matched for BMI, significantly higher concentrations of leptin were quantified in the plasma of AA patients (Fig. 3A). A positive and significant correlation was observed between circulating leptin levels with IL-5, IL-6 and IL-17 concentrations released by AA-derived activated T cell cultures (Fig. 3B). Interestingly, among patients, the leptin levels correlated inversely with the ability of 1,25(OH)2D3 to inhibit the *in vitro* production of IFN-γ, IL-6, IL-5 and IL-17 (Fig. 3C). On the other hand, the ability of the active form of vitamin D to elevate *in vitro* IL-10 production was inversely correlated with leptin levels (Fig. 3C). No correlation was observed with regard to IL-4 production and this adipokine.

3.4. Relationship between plasma levels of leptin and 1,25(OH)2D3 effects on cytokine secretion by $CD4^+$ T cells and IgE production by B cells in AA patients

AA may be mediated not only by Th2 cells but also by the Th17 phenotype [29–32], and similar to what was observed in PBMC cultures, the production of IL-4, IL-5, IL-6 and IL-17 by activated $CD4^+$ T cells were higher in patients than in the control group (Fig. 4A). In this system, the secretion of IL-10 (Fig. 4A) and IFN-γ (data not shown) by mitogen-stimulated $CD4^+$ T cells was lower in patients (Fig. 4A). Moreover, the highest levels of IL-5, IL-6 and IL-17 were detected among overweight/obese patients with severe AA (data not shown). Notably, no cytokine production was observed in unstimulated cultures.

Previous results displayed a lower efficiency of 1,25(OH)2D3 in controlling the overproduction of cytokines related to Th2 and Th17 cell phenotypes in PBMC cultures from AA patients. Here, even in the presence of highest concentration (20 ng/mL), 1,25(OH)2D3 was less efficient at reducing the production of IL-4, IL-5, IL-6 and IL-17 in samples from AA patients (Fig. 4A). Additionally, in these cultures, the active form of vitamin D was also less efficient at increasing IL-10 production (Fig. 4A). As demonstrated in the Fig. 4B, dramatic resistance to the *in vitro* vitamin D effects was seem among overweight/obese patients with severe AA.

With regard to the leptin, a positive and significant correlation was observed between this adipokine and IL-4, IL-5, IL-6 and IL-17 production by $CD4^+$ T cells in AA patients (Fig. 4C). On the other hand, lower IL-10 levels were oberved in cell cultures from patients with high plasma levels of leptin (Fig. 4C). Finally, the ability of 1,25(OH)2D3 in reducing pro-inflammatory cytokines and increasing IL-10 secretion in these cultures was inversely correlated with leptin levels (Fig. 4D).

Classically, in most patients, exacerbation of allergic asthma is accompanied by an increase in IgE production [21–23]. Here, IgE production in SEB-activated $CD4^+$ T/B cells co-cultures was higher in AA subjects when compared with the control group (Fig. 5A). Vitamin D, at the highest concentration (20 ng/mL), was less also efficient at reducing the production of this antibody in patient samples (Fig. 5A). Although the titers of IgE was not correlated with the clinical status (Fig. 5B), B cells from overweight patients trend to secrete more IgE than lean ones (Fig. 5C). No correlation was observed between plasma leptin levels and the *in vitro* production of IgE (Fig. 5D).

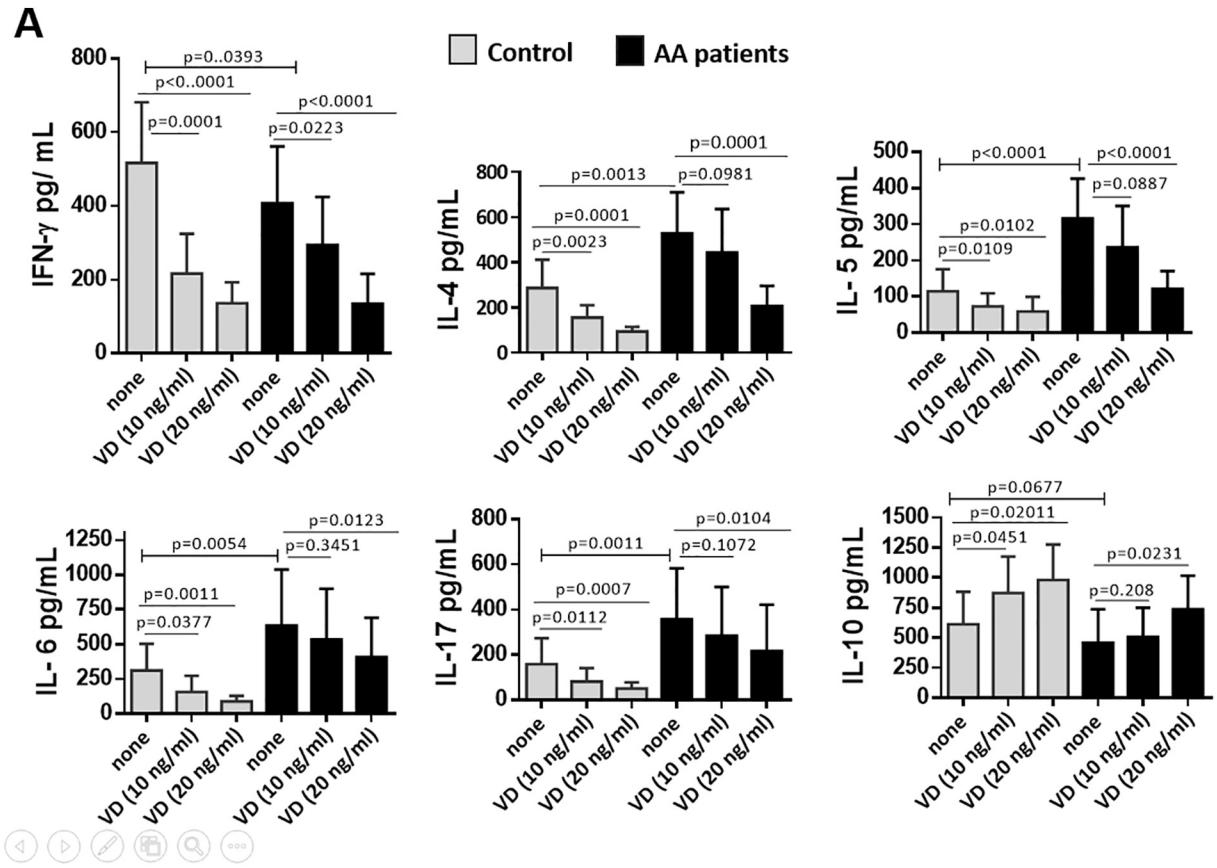
4. Discussion

The prevalence of allergic asthma (AA) has increased dramatically over the last three decades around the world. A similar scenario has been observed for obesity. According to the World Health Organization, in 2016, > 1.9 billion adults were overweight, and, of those, over 650 million were obese (WHO, 2018). Obesity has been described as an important environmental factor that plays a key role in the development and severity of the asthma [34–36]. In addition, obese AA patients account for significant health care expenditures as many of them respond poorly to commonly available therapies. Our findings indicate that being overweight/obese favors the expansion of $CD4^+$ T cells that produce cytokines implicated in the pathogenesis of AA. Furthermore, the immunomodulatory actions of vitamin D on the production of both T cell-derived cytokines and IgE was altered when individuals were overweight/obese and have higher circulating levels of leptin. To our knowledge, this is the first report showing a relationship between obesity, plasma leptin and the *in vitro* effects of vitamin D on AA patients.

In the present study, the majority of AA patients were women, which was expected since the disease is more common in females [66]. It is noteworthy that patients with severe asthma were overweight ($n = 4$) or obese ($n = 16$), and this was related to cytokine imbalance and hypo-responsiveness to vitamin D in $CD4^+$ T cell cultures.

Classically, AA is mediated by Th2 cytokines, such as IL-4 and IL-5, which coordinate an immune response involving allergen-specific IgE, mast cells and eosinophils [9,10]. These cytokines, along with lipid mediators of inflammation released by IgE-activated eosinophils, promote mucus overproduction and bronchoconstriction [21,22]. In the present study, and in line with the classical literature, higher IL-4 and IL-5 levels were detected in the supernatants of both PBMC cultures containing activated T cells and purified $CD4^+$ T cells from AA patients when compared with the control group. Although higher titers of IgE had been secreted in $CD4^+$ T cells/B cells co-cultures from AA patients, their levels were not associated with clinical status. There are at least three reasons to explain why *in vitro* IgE levels were not associated with disease severity. Firstly, as did not measured allergen-specific IgE, it is possible that its levels increase only during an acute asthma episode; secondly, specific IgE can be synthesized and produced locally by B cells within the respiratory mucosa [67]; and thirdly, not all cases of AA involve the production of Th2-related cytokines and detectable IgE. Indeed, allergic asthma is recognized as a heterogeneous disease with different inflammatory profiles. It is well known that Th17 cells can also mediate asthma, and in this case, it tends to be more severe, and is poorly controlled by inhaled corticoids (ICs) and beta-2-adrenergic agonists [68].

An increased IL-17 level has been reported in the peripheral blood



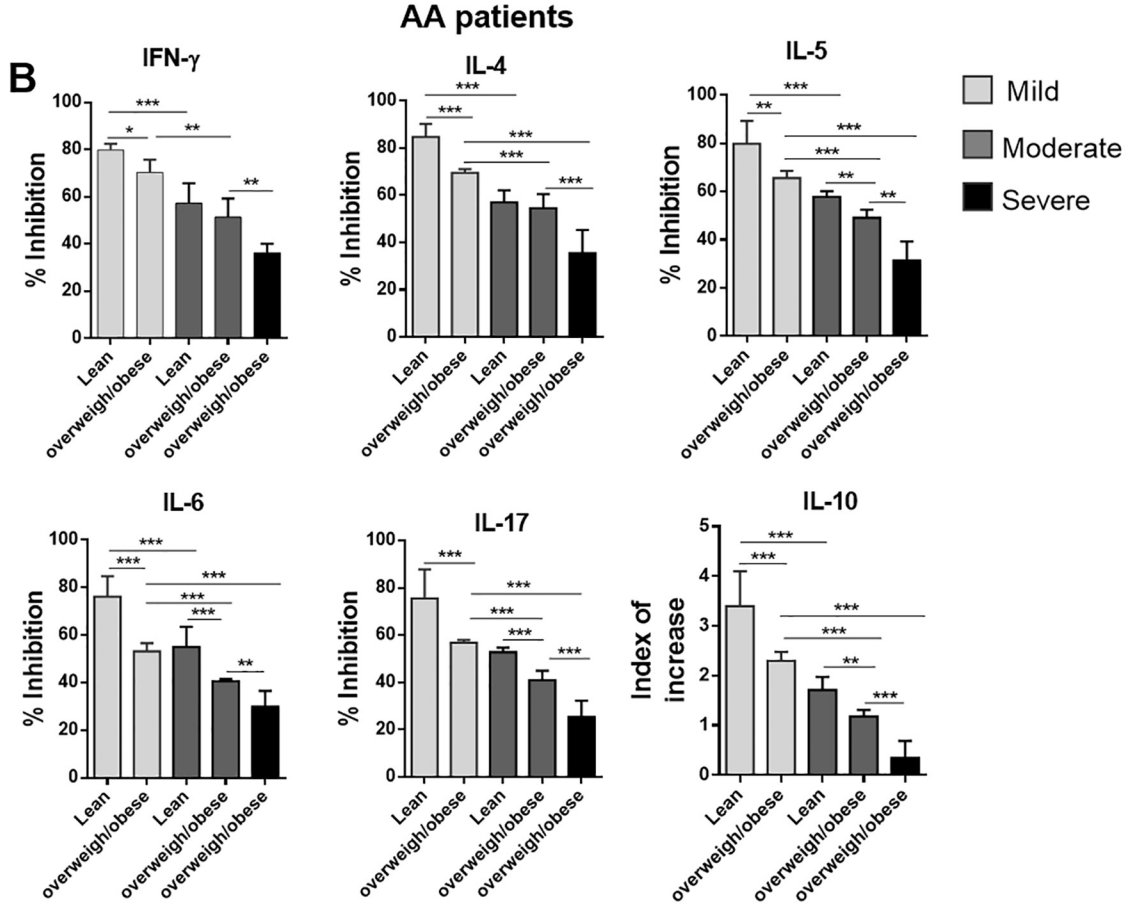


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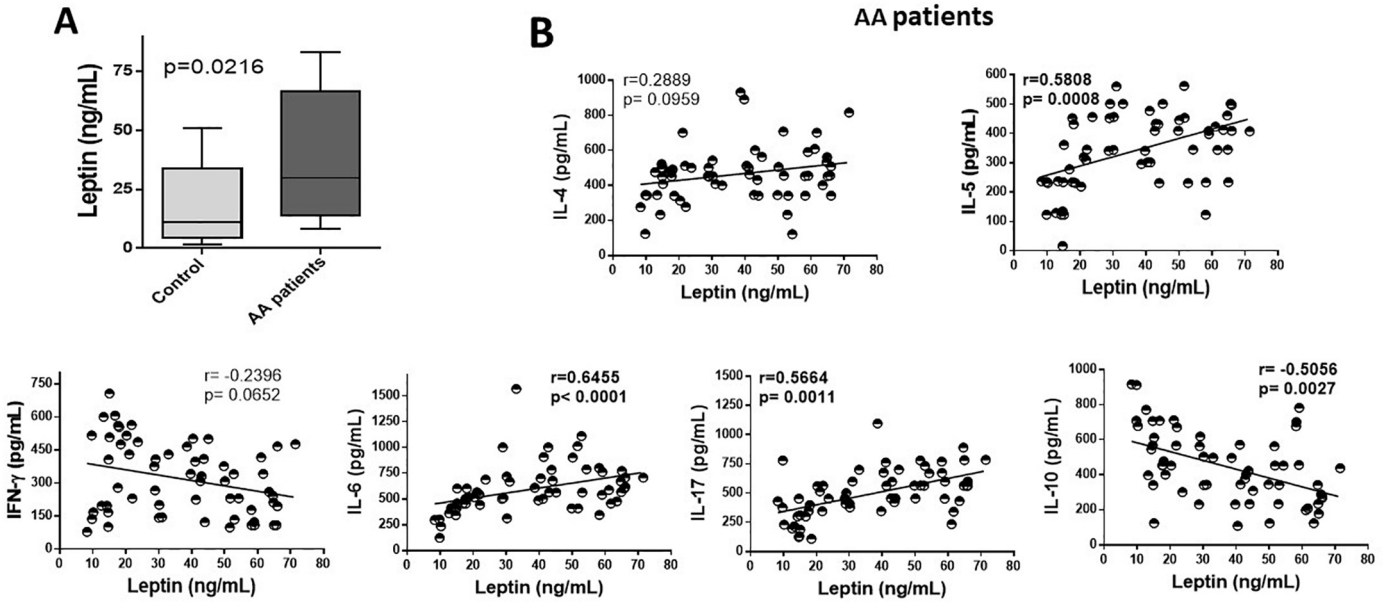
Fig. 2. Impact of 1,25 (OH)2D3 on the *in vitro* T cell-derived cytokine production of asthmatic patients. In (**A**), the PBMC cultures (1×10^6/mL) from asthmatic patients ($n = 60$) and control group ($n = 60$) were stimulated with anti-CD3/anti-CD28 beads (10 μL/mL), for 3 days. In some wells, different concentrations of 1,25 (OH)2D3 (10 and 20 ng/mL) were added at the beginning of cell cultures. In (**B**), the capacity of 1,25 (OH)2D3 (20 ng/mL) in modulating the release of cytokines by activated T cells from AA patient is showed in function of BMI and clinical status. The levels of cytokines were determined by ELISA. The mean values were compared and the p values shown in the fig. A. In B, (*), (**) and (***) indicates $p < .05$, $p < .01$ and $p < .0001$, respectively.

and lungs of patients with severe asthma [28]. By inducing IL-8 and TNF-α production, IL-17 favors the recruitment and activation of fibroblast and neutrophilic granulocytes to the lower airway [33,34]. The massive infiltration of neutrophils in the lung is a well-known clinical feature of steroid resistant asthma [29,30]. IL-17 also directly affects the airway smooth muscle leading to allergen-induced airway hyperresponsiveness [69]. In the present study, both activated T cells and purified $CD4^+$ T cells from AA patients produced elevated quantities of Th17-related cytokines IL-17 and IL-6 when compared with the control group. The serum IL-6, another Th17-related cytokine, is a marker of asthma severity [70]. In addition, we observed a relationship between asthma severity and IL-17 and IL-6 cytokines produced by T cells. The highest levels of IL-17 were detected in 14 from 20 AA patients with severe AA, and 11 of them were obese. On the other hand, IL-4 was



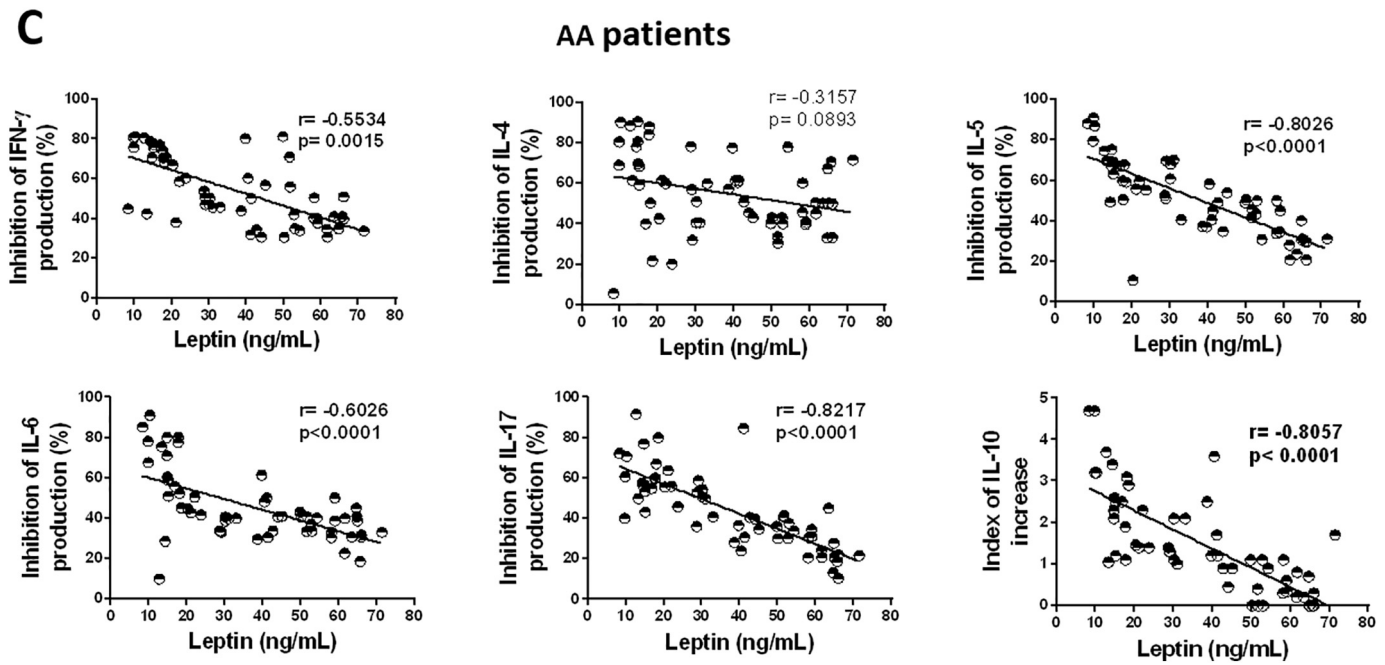


Fig. 3. Leptin dosage and its correlation with *in vitro* cytokine production by activated T cells from asthmatic patients in response to 1,25 (OH)2D3. In (**A**), the plasma levels of leptin were dosed in the control group ($n = 60$) and asthmatic subjects ($n = 60$). The concentrations of leptin were correlated with *in vitro* cytokine production (**B**) and the ability of 1,25 (OH)2D3 (20 ng/mL) (**C**) to modulate the release of those cytokines by T cells from AA patient following activation with anti-CD3/anti-CD28 beads (10 μL/mL). Both leptin and cytokine quantification were determined by ELISA.

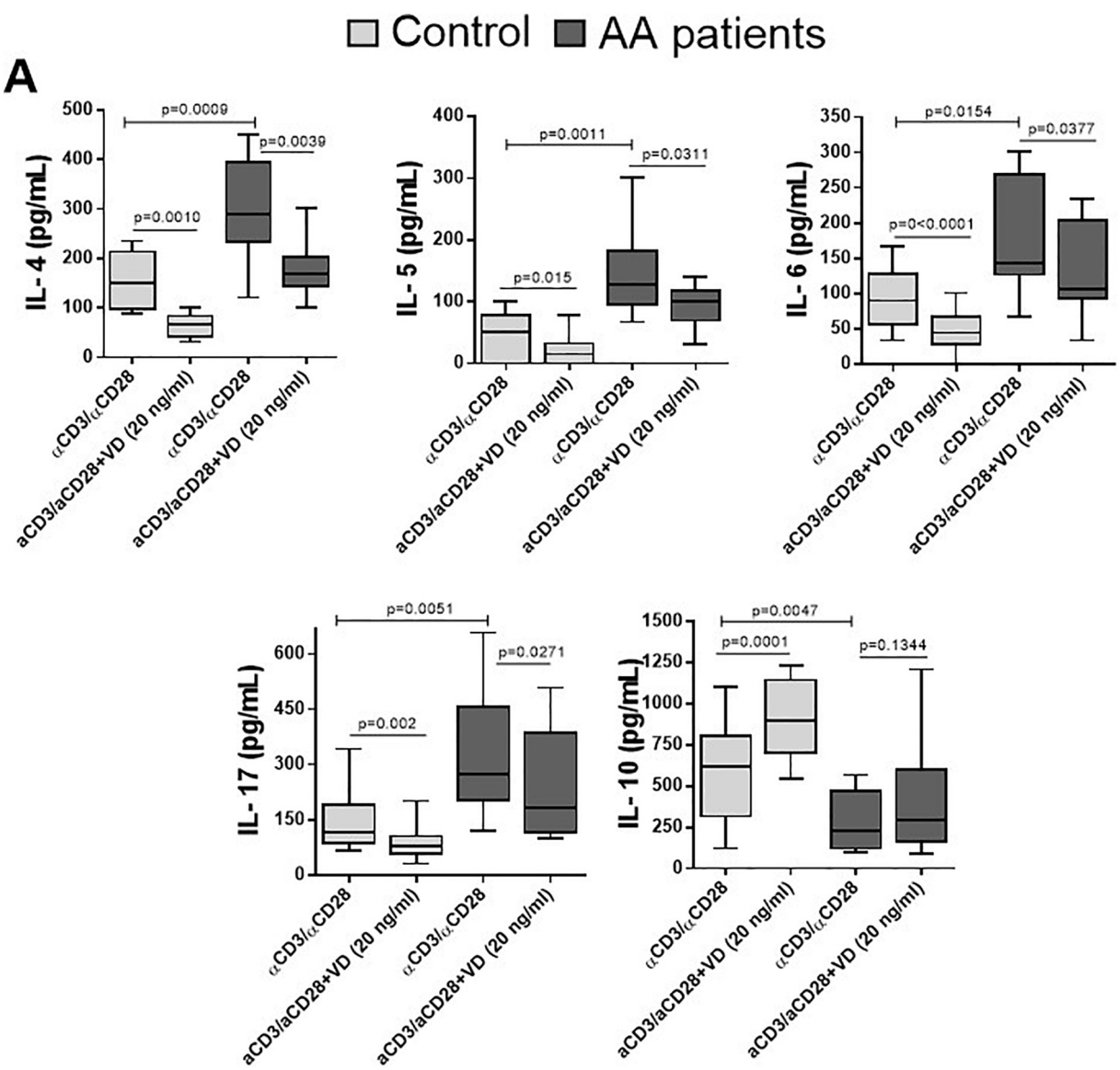


Fig. 4. Effects of 1,25(OH)2D3 on CD4$^+$ T cell-derived cytokine production by asthmatic patients with different plasma leptin levels. In (**A**), CD4$^+$ T (1×10^6/mL) from both control group ($n = 40$) and asthmatic patients ($n = 44$) were anti-CD3/anti-CD28 beads (10 μL/mL) for 3 days in the presence of 20 ng/mL of 1,25(OH)2D3. The levels of cytokines were determined by ELISA. In (B), all cytokines were stratified in function of obesity and clinical status of AA patients [mild (10 leans, 6 overweight and 2 obese), moderate (6 leans, 5 overweight and 5 obese) and severe (5 overweight and 5 obese)]. The correlation between *in vivo* leptin levels with (C) cytokine production and (D) the ability of 1,25(OH)2D3 at modulating cytokine secretion is also showed at the figures.

dominant among the other 6 patients with severe AA. Although the current literature affirms the existence of at least 2 asthma subtypes, Th2 and Th17 patterns, the levels of IL-5 in our present study varied widely among all severe AA patients. Nevertheless, mean values for IL-5 were invariably higher than those quantified in the cell cultures from patients with mild and moderate AA. More recently, some studies have also described a mixed Th2/Th17 pattern in some AA patients [31,32]. This phenotype is characterized by concomitant production of IL-5 and IL-17 cytokines, pulmonary infiltration of eosinophils, worse lung function and increased steroid requirement [31,32]. Unfortunately, the techniques used in the present study do not allow us to determine the presence of these hybrid Th2/Th17 cells, but this issue will be evaluated by our group using flow cytometry.

An interesting finding was with respect to IFN-γ production. This cytokine is classically secreted by Th1 and Tc-1 cells, both T cell phenotypes mainly implicated in protection against intracellular microorganisms and viruses [71,72]. The production of IFN-γ was significantly lower in AA patients. This phenomenon was particularly evident among overweight/obese patients with severe asthma. In addition to structural changes patients with severe AA favor infections of the lower airways. This is probably due to the decreased capacity of T cell to produce IFN-γ which increases susceptibility to respiratory viral infection, such as influenza and rhinovirus [73]. Increased risks of pneumococcal pneumonia and invasive pneumococcal disease has also been reported in AA patients [74]. The lower serotype-specific pneumococcal antibody titers in these patients were related to an elevated IL-5 to IFN-γ ratio secreted by PBMC after stimulation by allergens [75]. Moreover, those patients show a significantly higher risk of community acquired *E. coli* infection [76]. Altogether, these findings reveal a negative impact of AA on immune resistance against pathogens,wis worsened by obesity.

When obesity occurs with asthma, it is associated with more severe asthma, poor pharmacological control and increased asthma exacerbation [34–36]. This deleterious relationship is probably due to the generation of unique inflammation mediators that modulate the behavior of T cell phenotypes involved in asthma. It is known that obesity

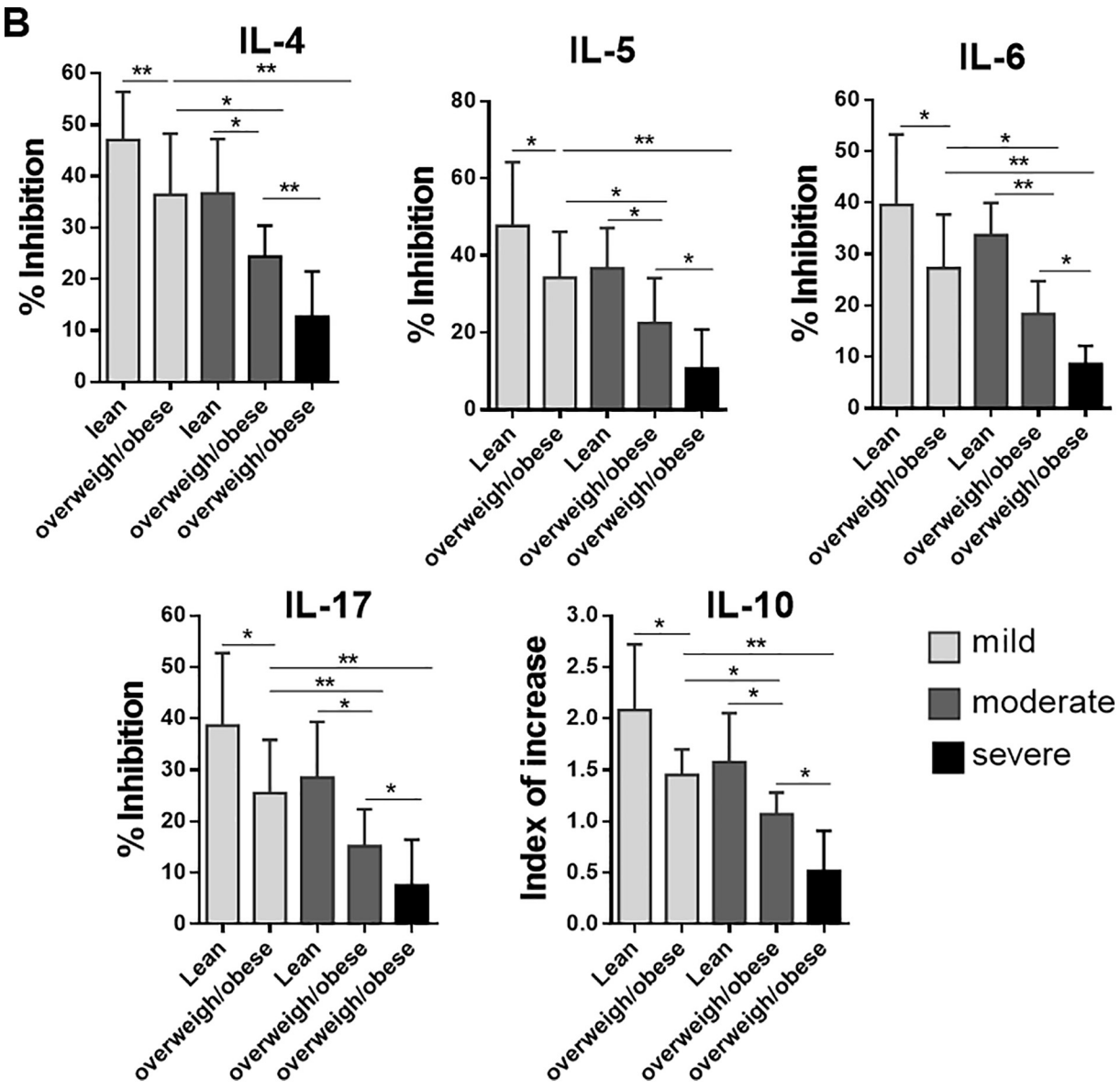


Fig. 4. (*continued*)

complicates both Th2 and Th17-associated asthma, and the deleterious impact of weight gain on allergies may be linked to overproduction of leptin.

Elevated expression of leptin receptors has been observed on T cells from obese subjects [41,42]. On $CD4^+$ T cells, this adipokine favors the expansion of effector T cells, like Th17 cells [40,43–48]. On the other hand, high leptin levels damage regulatory T cell function by inducing IL-1β, TNF-α and IL-6 production [77]. Leptin effects can be direct or indirect, by up regulating the secretion of pro-inflammatory cytokines (TNF-α, IL-6, IL-12) and the expression of co-stimulator molecules by dendritic cells [78]. These immunogenic antigen presenting cells are involved in the induction of hypersensitivity disorders [79,80].

In our study, occurrence of obesity favors an increase of IL-6 production by activated T cells in the control group. In the asthma group, the release of IL-6, IL-17, IL-4 and IL-5 by whole T cells and $CD4^+$ T cells were significantly higher in overweight/obese patients. Interestingly, although both experimental groups were matched for BMI, significantly higher leptin levels were quantified in the plasma of AA patients. This could be explained by elevated inflammatory status in those patients in comparison with the control group, since pro-inflammatory cytokines enhance leptin production [81]. A positive and significant correlation was observed between levels of this adipokine and the concentrations of IL-5, IL-6 and IL-17 quantified in the supernatants of PBMC containing activated T cells as well as purified $CD4^+$ T cell cultures. By contrast, a negative correlation was observed between *in vivo* leptin and IL-10 produced by $CD4^+$ T cells. This latest finding is in line with the literature that correlates hypersensitive disorders, like asthma, with failures in regulatory mechanisms mediated by regulatory T cells, such as IL-10 production [82,83].

In the present study, although no difference was observed concerning IL-10 released by T cells from AA patients from different clinical subgroups, the levels of this anti-inflammatory cytokine were lower in overweight/obese individuals. Interestingly, the proportion of IL-10-secreting $CD4^+$ T cells was significantly lower in overweight/obese patients with severe asthma. This result suggests that obesity may negatively affect IL-10 synthesis by $CD4^+$ T cells in patients with severe asthma. Knowledge about the role of regulatory T cells in allergy comes from immunotherapy (AIT) studies [84]. Successful AIT has been associated with up regulation of functional Tregs and Tr-1 cell subsets, all of them able to produce high levels of anti-inflammatory cytokines, such as IL-10 and TGF-β [85–88]. IL-10 and TGF-β inhibit IgE released by decreasing the production of Th2 cytokines [89], inhibiting the

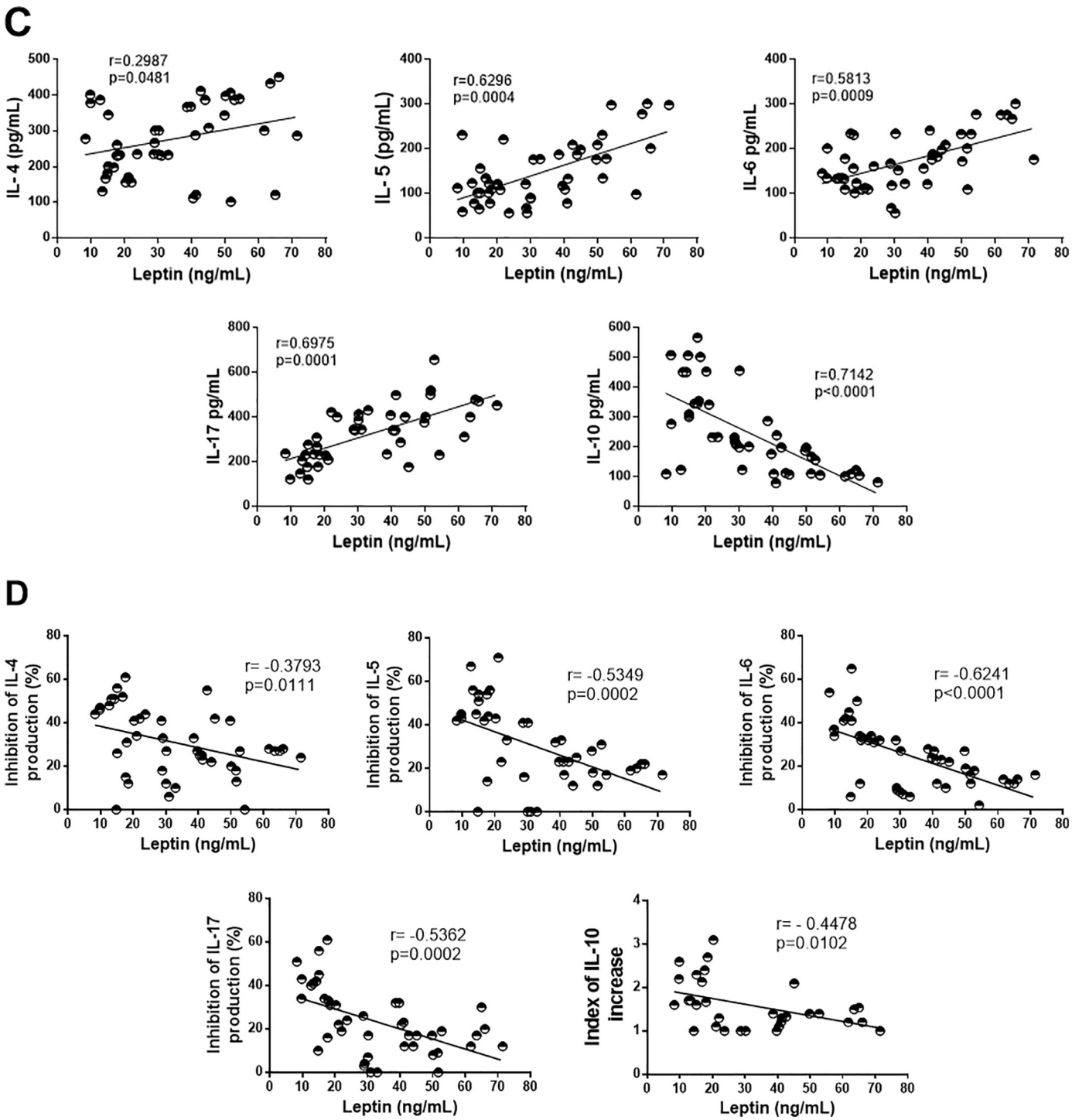


Fig. 4. (*continued*)

degranulation of effector mast cells and eosinophils [90–92]. This is associated with downregulation of FcεRI expression on these cells [84,93,94], and modulating allergen-driven effector T h17 cells as well as neutrophil activation.

Disturbances in regulatory mechanisms in allergic asthma could also be associated with vitamin D deficiency. Although vitamin D deficiency is common in the general population, obese individuals present a higher risk of developing it than eutrophic individuals [50]. Vitamin D deficiency has been reported to be associated with greater risk of asthma [52–59]. Additionally, low serum 25(OH) vitamin D levels increase corticosteroid requirements for controlling inflammation during asthmatic episodes [95]. Further, a study by Zhang et al. [96] demonstrated the ability of the active form of vitamin D, 1,25(OH)2D3, to enhance corticoid's capacity to elevate the production of IL-10 by LPS-activated monocytes from asthmatic patients. 1,25(OH)2D3 also potentiates the ability of corticoid to generate IL-10-producing Tr1 cells [97,98]. These induced Tr1 cells are able to impair proliferation and cytokine production by human effector T cells, including allergen-specific Th2 cell lines [99]. Moreover, 1,25(OH)2D3 inhibits *in vitro* Th17-related cytokine production in severe asthmatic patients regardless of their clinical responsiveness to corticosteroids [100]. In the present study, the active form of vitamin D was less effective at controlling the

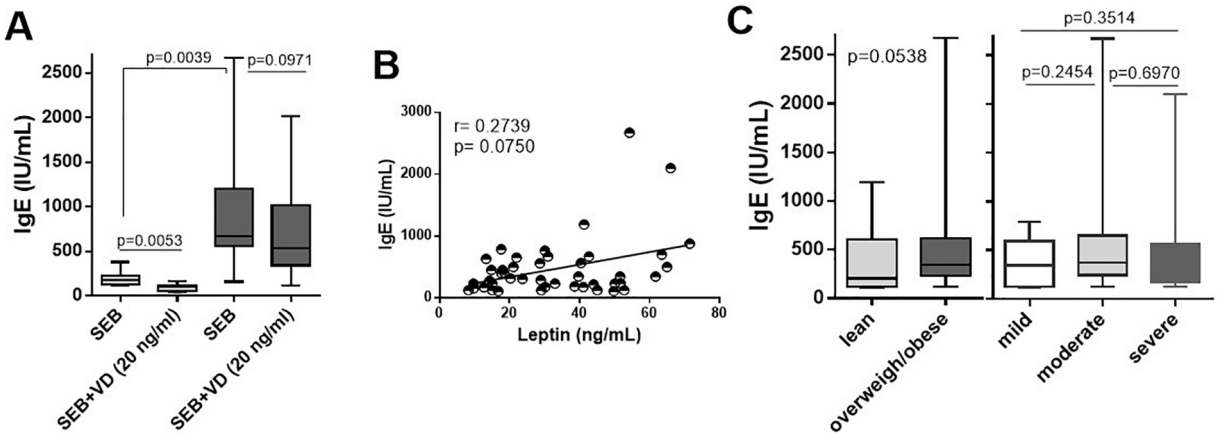


Fig. 5. The *in vitro* IgE production in allergic asthma patients in the presence of vitamin D. In (**A**), $CD4^+$ T (1×10^4/500 μL) and B cell (1×10^4/500 μL) co-cultures from control ($n = 40$) and asthmatic patients (n = 44) were stimulated with SEB (1 μg/mL) for 6 days in the presence of 20 ng/mL of 1,25(OH)2D3. The IgE contents in the supernatants were dosed by ELISA. Among patients with mild (10 leans, 6 overweight and 2 obese), moderate (6 leans, 5 overweight and 5 obese) and severe (5 overweight and 5 obese) asthma, IgE titers are showed in function of either leptin levels (**B**) or obesity and clinical status (**C**).

in vitro overproduction of Th2- and Th17-related cytokines in activated PBMC and $CD4^+$ T cell cultures from AA patients in comparison with the control group. Higher *in vitro* 1,25(OH)2D3 resistance was observed in cell cultures from overweight/obese patients with severe asthma. Moreover, in AA patients, the active form of vitamin D was also less efficient at elevating *in vitro* IL-10 production, with this effect being more marked in activated $CD4^+$ T cells. Finally, the circulating leptin levels were inversely correlated with the ability of 1,25(OH)2D3 to diminish pro-inflammatory cytokines and increase IL-10 released by $CD4^+$ T cell cultures from AA subjects.

5. Conclusions

In summary, our findings suggested that obesity negatively impacts the clinical course of allergic asthma by favoring an imbalance between Th2/Th17 phenotypes and regulatory T cell ratio. These deleterious effects may at least in part be due to lower $CD4^+$ T cell responsiveness to vitamin D and higher circulating levels of leptin.

Declaration of competing interests

All authors declare that there are no conflicts of interest.

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