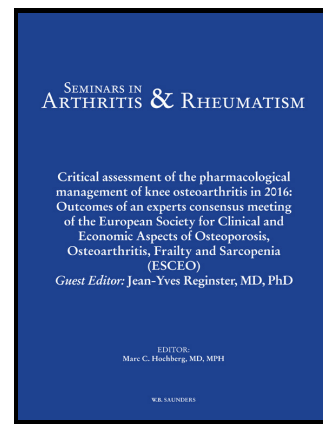


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VITAMIN D AND ANTIPHOSPHOLIPID SYNDROME: A RETROSPECTIVE COHORT STUDY AND META-ANALYSIS

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ABSTRACT

Objectives: a) To determine serum 25-OH vitamin D (vitD) levels in primary antiphospholipid syndrome (APS) and to compare them with patients with positive antiphospholipid serology who do not meet clinical criteria for APS, and with healthy controls. b) To analyze the association of vitD levels with both the clinical manifestations and the immunological profile of patients with primary APS. c) To perform a meta-analysis evaluating potential differences in serum vitD levels between APS and controls as well as the frequency of vit D deficiency in APS patients.

Methods: Retrospective study including 74 patients with primary APS, 54 with positive antiphospholipid (aPL) serology not meeting clinical criteria for APS and 215 healthy controls. We considered 30 ng/ml and 10 ng/ml as the thresholds for vitamin D insufficiency and deficiency, respectively. Meta-analysis included 4 case-control studies (325 primary APS patients and 507 controls) and was conducted by fitting random effects models and checked for heterogeneity.

Results: Median serum vitD levels were similar in the three groups: 21 ng/ml in primary APS, 25 ng/ml in the aPL-positive group, and 21 ng/ml in controls ($p=0.115$). However, we found differences in the PTH levels, being 40.4 ± 24.9 pg/ml in APS, 34.1 ± 18.2 pg/ml in aPL serology and 23.4 ± 12.6 pg/ml in healthy controls ($p<0.001$). Regarding vitamin D deficiency, we found significant differences across the groups: 16.2% in APS, 11.1% in patients with positive serology and 3.7% in controls ($p=0.001$). There was a trend for the presence of thrombotic events in patients with vitamin D deficiency (38.9% vs 19.1%, $p=0.071$). The meta-analysis confirmed that the combined mean difference in serum vitD levels between APS and controls was -3.605 ($p < 0.001$) and that APS patients had an increased frequency of vitD deficiency, with an OR 3.06 (95% CI 2.12 - 4.43, $p<0.001$).

Conclusions: APS patients show higher frequency of vitD deficiency than the healthy individuals. The meta-analysis study, including three cohorts and ours, suggests that APS patients have significantly lower serum vitD levels and higher frequency of vitD deficiency than controls.

Key Words: vitamin D, antiphospholipid syndrome, meta-analysis

1. INTRODUCTION

Antiphospholipid syndrome (APS) is an autoimmune disease characterized by the presence of antiphospholipid antibodies (aPL) and at least a clinical event defined as thrombosis and/or pregnancy morbidity [1] APS may also appear associated with an underlying systemic autoimmune rheumatic disease. The prevalence of aPL in the general population is around 5% [2]. Transiently positive serology may appear in some infections and in any inflammatory state [2;3]. In patients with systemic lupus erythematosus (SLE), aPL are present in up to 41% even in the absence of APS [4]. Vitamin D (VitD) has a very well known role in bone metabolism and calcium homeostasis, having also important immunomodulatory functions [5]. Its deficiency has been linked to the pathogenesis and clinical manifestations of autoimmune diseases such as diabetes, multiple sclerosis, Crohn disease, rheumatoid arthritis, SLE or Sjögren syndrome [6-9].

Moreover, in vitro models have shown that vitD inhibits the expression of tissue factor (TF) in monocytes stimulated by anti-beta 2 glycoprotein I (aB2GPI) antibodies derived from APS, suggesting a potential role of vitD in the pathogenesis of the disease [10]. Some case-control studies have reported low serum levels of vitD in APS patients as well as high frequency of vitD deficiency [10-14]. However, some of those studies lack of a control population, include small samples and none of them evaluate patients with isolated aPL serology without fulfilling the clinical criteria for APS.

The aims of the present study were a) to determine serum vitD levels in patients with primary APS and to compare them with patients with positive aPL who do not meet clinical criteria for APS, and with healthy controls, b) to analyze the association of the vitD levels with both the clinical manifestations and the immunological profile of patients with primary APS and c) to perform a metaanalysis evaluating potential differences in serum vitD levels between APS and controls as well as the frequency of vitD deficiency in APS patients.

2. MATERIAL AND METHODS

2.1 Study population

We conducted a retrospective study including patients attending the Rheumatology clinic from a tertiary center in Northern Spain from January 2007 to December 2013. We included 74 patients with primary APS (all of them met Sidney classification criteria [1]), 54 with positive aPL serology not meeting clinical criteria for APS and 215 healthy controls consisting of voluntary adults from the general population attending a primary care center. They were frequency matched for age and sex. None of the patients met the ACR classification criteria for SLE or other concomitant autoimmune disease. In order to evaluate seasonal variations of serum vitD levels, patients and controls were grouped according to two time points: sunny season (July to October) and dark season (November to June).

2.2 Laboratory tests

IgG and IgM anticardiolipin (aCL) and aB2GPI levels were measured in real time in our routine by ELISA (Aesku diagnostics, Wensdelsheim, Germany) and expressed in GPL or MPL units or U/ml, respectively. Titers were considered to be positive when they were above the 99th percentile, thus corresponding to 20 GPL, MPL

or U/ml. We considered positive those titers reported as medium (20-30) or high (>30), being low titers considered as negative.

Lupus anticoagulant was determined according to the standard guidelines Subcommittee on Lupus Anticoagulant/antiphospholipid antibody (scientific and standardization Committee of the International Society of Thrombosis and Haemostasis)[15].

Samples from routine aPL tests were frozen at -80°C and used to measure in a single run 25-OH vitamin D and intact PTH. Serum 25-OH vitamin D concentration was measured by automated competitive chemiluminiscence assay (Liaison XL, DiaSorin Inc, Stillwater MN USA). Our laboratory is DEQAS (Vitamin D External Quality Assessment Scheme). Minimum detectable concentration was estimated to be 4 ng/ml. We considered 30 ng/ml and 10 ng/ml as the thresholds for vitamin D insufficiency and deficiency, respectively [16;17]. Total intact PTH was measured by specific automated chemiluminiscence assay (iSYS, IDS-iSYS Multi-Discipline Automated Analyser, Pouilly-en Auxois, France). The sensitivity of the PTH assay was 5 pg/ml. Normal values were considered as below 45 pg/ml.

2.3 The meta-analysis

A comprehensive search of PubMed and Cochrane database was completed and supplemented by hand searching of the references of all selected articles. "Vitamin D and antiphospholipid syndrome" without restriction to any language published up to March 2017. This meta-analysis followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [18].

Studies that included patients with APS and vitD were reviewed as shown in the flow chart. **Figure 1.** Information was collected on study design, study sample, characteristics of the study population, serum vitD levels and vitD deficiency definition.

2.4 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows version 15 (Armonk, NY: IBM Corp.). Median and ranges or mean values and standard deviations were calculated for the continuous variables. Between-group differences were analyzed using the Student t-test, ANOVA or the chi square tests. A 2-tailed $p < 0.05$ was considered statistically significant.

Meta-analyses were conducted by fitting random effects models and were checked for publication bias by funnel plots as shown in supplementary Figure 1 [19]. I^2 statistic was calculated to quantify the proportion of the total variation owing the heterogeneity [20] Statistics were performed using Comprehensive Meta-analysis, version 2.0 (Biostat, Englewood, NJ, USA) [21].

3. RESULTS

The main clinical and laboratory features of the study population are summarized in the **table 1**.

Table 1. Main clinical and immunological features of the study population

| Variable | APS (n=74) | aPL serology (n=54) | Healthy controls (215) | p |
|--------------------------------------|-----------------|---------------------|------------------------|-------|
| Age, yrs (mean \pm SD) | 46.1 \pm 15.3 | 49.2 \pm 17.2 | 49.8 \pm 8.9 | 0.08 |
| Women, n (%) | 65 (90.3) | 42 (84) | 175 (81.4) | 0.210 |
| Smoking, n (%) | 30 (40.5) | 16 (30.2) | ND | 0.27 |
| Hypertension, n (%) | 24 (32.4) | 13 (24.5) | ND | 0.43 |
| Dyslipidemia, n (%) | 22 (29.7) | 8 (15.1) | ND | 0.06 |
| Diabetes, n (%) | 6 (8.1) | 2 (3.8) | ND | 0.47 |
| Clinical manifestations, n (%) | | | | |
| Thrombotic APS | 34 (45.9) | NA | NA | - |
| Obstetric APS | 37 (50) | | | |
| Mixed APS | 3 (4.1) | | | |
| Positive aCL antibodies | 57 (77) | 48(90.6) | NA | 0.06 |
| Positive aB2GPI antibodies, n (%) | 49 (66.2) | 37 (69.8) | NA | 0.71 |
| Positive lupus anticoagulant, n (%) | 23 (54.8) | 15 (44.1) | NA | 0.49 |
| Number of positive antibodies, n (%) | | | | |

| | | | | |
|--|---------------------------------|--------------------------------------|---|-------|
| 1 | 29 (39.2) | 18 (33.3) | | |
| 2 | 27 (36.5) | 22 (40.7) | NA | 0.71 |
| 3 | 18 (24.3) | 14 (25.9) | | |
| 25 OH D ng/ml, Mean (SD) Median (IR) | 21.99 (11.8) 20.95 (14-27.4) | 25.25 (10.9) 25.0 (18.6- 32.3) | 24.74 (12.8) 21.0 (15.6- 31.4) | 0.205 |

Abbreviations: APS: antiphospholipid syndrome, aPL: antiphospholipid, aCL: anti-cardiolipin, aBGPI: anti-beta 2 glycoprotein I, NA: non-applicable, SD: standard deviation, IR: interquartile range.

Overall, 20.5% of measurements were performed during the sunny season. As expected, higher levels of vitD were found in this period (median 25 ng/ml; range 10-69 vs 20 ng/ml; range 4-105, $p < 0.001$). Seasonal variation was observed in both APS and controls, but no statistically significant differences were observed in the aPL serology group.

Median levels of vitD were similar in the three groups: 21 ng/ml (range 5-69) in primary APS, 25 ng/ml (4-50) in the aPL-positive group, and 21 ng/ml (7-105) in healthy controls ($p=0.115$). However, we found differences concerning serum PTH levels, being 40.4 ± 24.9 pg/ml in APS, 34.1 ± 18.2 pg/ml in aPL serology and 23.4 ± 12.6 pg/ml in controls ($p < 0.001$). *Post hoc* analysis confirmed the differences in PTH levels between both APS and aPL with controls.

As shown in **Figure 2**, we found no significant differences in the frequency of vitD insufficiency among the three groups ($p=0.222$). However, there were differences in the prevalence of vitD deficiency across the groups ($p=0.001$)

APS patients were classified as thrombotic and obstetric phenotype. We found no differences in serum vitD levels according to these clinical phenotype (median 19 ng/ml; range 6-44 in thrombotic APS vs 22 ng/ml; range 6-69 in obstetric APS; $p=0.292$), neither globally nor stratifying by season. There was no significant association between vitD insufficiency and the presence of thrombotic or obstetric

events. Nevertheless, we found a trend for the presence of more thrombotic events in patients with vitamin D deficiency (38.9% vs 19.1%; $p=0.071$).

Regarding the autoantibodies profile, we found no association between vitD insufficiency and the number of positive antibodies, or the presence of either aCL or aB2GPI antibodies. However, we found an association between vitD insufficiency and the presence of lupus anticoagulant (54.7 % vs 18.2%, $p=0.047$). When the analysis was restricted to APS patients, we found an association between vitD insufficiency and higher number of positive antibodies and positivity for aB2GPI ($p=0.050$ and $p=0.023$, respectively). No association was found between vitD deficiency and the immunological profile.

In line with traditional cardiovascular risk factors and serum vitD levels, we found a trend between dyslipidemia and vitD insufficiency (27.8% vs 10.3%, $p=0.080$) that persisted when we restricted the analysis to the aPL serology group (21.6% vs 0%, $p=0.088$). There was also a trend for an association between insufficient vit D levels and hypertension (32.4 % vs 6.3%, $p=0.079$) in the aPL serology group. We also studied the association between traditional cardiovascular risk factors and vitD deficiency, with a tendency of association with diabetes mellitus (16.7% vs 4.6%, $p=0.085$), and with tobacco use when we restricted the analysis to the APS group (66.7% vs 35.5% , $p=0.058$).

Vitamin D levels are decreased in patients with APS

Only three studies had previously provided data on serum vitD levels in APS patients and controls. As detailed in **Table 2**, Agmon-Levin [10] et al., included both patients with primary and secondary APS, while the rest of authors included only primary APS patients.

The meta-analysis (**Figure 3a**) showed that the combined mean difference in vitD levels between APS and controls was -3.605 (SE 0.941, $p < 0.001$). No heterogeneity was detected in the studies analyzing mean serum vitD levels, I^2 0% (95%CI: 0.0 - 99.9%).

Vitamin D deficiency is more frequent in APS patients than in controls

Four studies provided data on the frequency of vitD deficiency in APS patients. As shown in the **table 2**, the threshold for vitD deficiency was not homogeneous. This was taking into account, as explained in the statistical analysis section. The metaanalysis of these four studies showed that APS patients have an increased frequency of vitD deficiency, with an OR 3.06 (95% CI 2.12 - 4.43, $p < 0.001$) (**Figure 3b**). There is some evidence suggestive of mild heterogeneity between the four studies; I^2 24.7% (95% CI: 0.0 - 90.3%).

Table 2. Main demographic and clinical characteristics of the patients reported in previous literature

| | Orbach 2007# [11] | Klack 2010# [12] | Paupitz 2010[13] | Agmon Levin 2010[10] | Andreoli 2012[14] | Riancho-Zarrabeitia [17] |
|----------------------------------|-------------------|------------------|-------------------|---|-------------------|--------------------------|
| Study design | Cohort | Cohort | Case controls | Case controls | Case controls | Case controls |
| Population | ND | Brazil | Brazil | European | Italian | Spanish |
| Number of patients | 160 | 46 | 23 | 179 (113 primary APS, 66 secondary APS) | 115 | 74 |
| Control group | No | No | 23 | 141 | 128 | 215 |
| Female sex, % | ND | 93.5 | 100 | 80 | 83 | 90 |
| Age, years, (mean \pm SD) | ND | 40.2 \pm 11.9 | 33 \pm 8.2 | ND | 46 [18-79] * | 46.1 \pm 15.3 |
| White race % | ND | 82.6 | 86.9 | ND | 96 | ND |
| 25 OH vitD, ng/ml, mean \pm SD | 11.9 \pm 5.4 | 23.3 \pm 10.5 | 21.64 \pm 11.26 | 16.7 \pm 9 | ND | 21.99 \pm 11.8 |
| Insufficiency threshold, ng/ml | - | 30 | - | 30 | 30 | 30 |
| Insufficient levels, % | ND | 74% | ND | 51.4% | 77% | 82% |
| Deficiency threshold | 20 | 10 | 20 | 15 | 10 | 10 |
| Deficient levels, % | ND | 11% | 56.5% | 49.5% | 17% | 16.2% |
| Venous thrombosis, % | ND | 63 | 82.6% | ND | 41 | 50% |
| Arterial | ND | 32.6 | 34.8 | ND | 35 | |

| | | | | | | |
|---------------------|----|------|------|----|----|----|
| thrombosis, % | | | | | | |
| Obstetric events, % | ND | 28.3 | 43.5 | ND | 36 | 54 |

Abbreviations: ND no data, # studies not included in the meta-analysis due to a lack of control group, * median [range]

4. DISCUSSION

It has been suggested that vitamin D may play a role in the pathogenesis of APS [8;11]. Furthermore, a few studies have found a higher frequency of vitamin D deficiency in APS [10;13;14]. In the present study, we included 74 patients with primary APS, 54 with a positive aPL serology and 215 healthy controls. There were no differences in serum vitD levels among the groups, but APS patients had higher frequency of vitD deficiency than aPL and controls. Moreover, we found a trend towards an association between vitD deficiency and the presence of thrombotic events. The meta-analysis confirmed that APS patients have lower levels of vitD and higher frequency of vitD deficiency than the control population.

Previous studies have reported lower serum vitD levels and higher frequency of vitD insufficiency in APS patients. However, in initial studies a control group was lacking (**Table 2**)[11;13]. Further studies, included a control population and confirmed that APS patients show a higher frequency of vitD insufficiency and/ or deficiency[10;13;14]. Regarding vitD serum levels, some authors found lower levels in APS patients [10;13] while others found differences only during the summer period but not in spring [14]. We did not find significant differences when comparing median serum vitD levels among the three groups. However, intact PTH levels were significantly higher in APS group, suggesting a reactive increase in response to lower levels of vitD [22-24]. Most of previous studies included only patients with primary APS. Nevertheless, Agmon et al. [10] also included patients with secondary APS. They showed that those with secondary APS had lower vitD levels than patients with primary APS, and both of them lower than controls. This fact could be explained by the sun avoidance and the use of sunscreens in patients with SLE the presence of renal disease, or the use of some medications such as steroids. Overall, the finding of low vitD levels in APS patients keeps in line with the suggested role of vitD in the pathogenesis of many autoimmune conditions such as rheumatoid arthritis, diabetes or multiple sclerosis [8]. In fact, it is

well known that vitamin D has regulatory properties in the immune system. Thus, it regulates the differentiation and activation of CD4⁺ lymphocytes, inhibits the differentiation of dendritic cells, as well as the differentiation from monocytes to macrophages, preventing them from releasing inflammatory cytokines and chemokines [11]. Besides, vitD deficiency is associated with impaired TLR function in aged subjects [5]. Moreover, it reduces IL-12 production and so, IL12-mediated Th1 responses and the production of IFN-gamma and IL-2 [25]. On the other hand, it increases IL-10 and promotes Th2 type T helper function. Regarding B cells, vitD has shown to inhibit antibody secretion and autoantibody production [26].

Regarding traditional cardiovascular risk factors, we found some trend, albeit non-significant, for an association between vitD insufficiency and both dyslipidemia and hypertension. Moreover, there was a trend towards an association between vitD deficiency, diabetes and tobacco use in APS and aPL patients. Additionally, there was a trend, although not statistically significant, for differences in the frequency of dyslipidemia between APS and aPL, and the overall frequency of patients with cardiovascular risk factors was low. Therefore, other studies in larger populations are needed in order to elucidate the interactions between vitD availability, traditional cardiovascular risk factors and cardiovascular outcomes. There is poor evidence about the association between traditional cardiovascular risk factors and vitD deficiency in APS patients. Paupitz et al [13] found that vitD was inversely correlated with fat mass and fat percentage, confirming the findings by Klack [12] et al., and previous studies that demonstrated low vitD levels in obese individuals. This fact is in line with the previous knowledge, that in general population, low vitD status is associated with insuline resistance, hypertension, hyperlipidemia and diabetes and even with cardiovascular events [27;28]. Besides, in SLE patients low vitD has been associated with hypertension, hyperlipidemia [29] and diabetes [30]. The proposed pathogenic mechanisms include the dysregulation of calcium and phosphate homeostasis in cells and interstitial space, and serum PTH increase secondary to vitD deficiency that is considered a risk factor for cardiovascular disease and a predictor of cardiovascular events (probably due to a calcium overloading of cardiomyocytes). However, even though most of the vitD receptor knockout animal models support a causative role between vitD and vascular health, data from randomized studies does not uniformly support the assumption of a causal relationship, suggesting the possibility that low serum vitamin D levels in cardiovascular disease is only an epiphenomenon or merely a

general marker of poor health [28]. Further studies are needed to elucidate this hypothesis.

When analyzing APS clinical manifestations, we found that patients with vitD deficiency tended to have more thrombotic events than non-deficient ones. This finding keeps in line with previous studies that suggested an association between vitD and thrombosis in cancer patients [31] and general population [32;33]. Moreover, Andreoli et al [14] found that APS women with thrombotic manifestations had lower levels of vitD than women with pure obstetric disease during the summer period. Agmon et al., [10] also found an association between lower serum vitD levels and thrombotic events, as well as an association with pulmonary and ophthalmologic manifestations, *livedo reticularis* and skin ulcerations. This could be explained by the fact that aPL induced activation of endothelial cells and monocytes results in overexpression of tissue factor and adhesion molecules that are considered thrombogenic mechanisms in APS [34]. Agmon et al., [10] showed that vitD inhibited in vitro the expression of tissue factor induced by aB2GPI antibodies, therefore exerting an anti-thrombotic effect. However in our study vit D was measured at a time that was not necessarily the time of thrombosis, thus, many other factors can interfere the results. The association of vitamin D and thrombosis should be assessed in well-designed studies and also at the time of the thrombotic event.

On the other hand, we did not find any association between vitD deficiency and obstetric outcomes. However, vitD deficiency has been linked with adverse pregnancy outcomes in general population [35-37]. Some of the pathogenic mechanisms suggest that vitD deficiency predisposes to a proinflammatory response, increases oxidative stress and leads to endothelial dysfunction. Moreover, vitD is thought to regulate genes responsible for trophoblast invasion and angiogenesis. There is very scarce information about the role of vitD and obstetric events in APS. Gysler et al., [38] suggested a combined treatment of low molecular weight heparin (LMWH) and vitD in pregnant women with APS, as they show that active vitD regulated aPL mediated inflammation and mitigate the release of antiangiogenic factors induced by LMWH.

We found an association between vitD insufficiency and lupus anticoagulant. When we restricted the analysis to APS patients, we found an association between aB2GPI and a higher number of positive antibodies and vitD insufficiency. This finding keeps in line with *in vitro* studies showing that active vitD reduced antibody secretion and autoantibody production in SLE patients [26]. Regarding APS patients, only Paupitz et al., [13] evaluated the autoantibodies profile and the vitD status. They found no correlation between serum 25OHD levels and aCL. A recent preliminary study [39] reported that in APS patients, vitamin D supplementation might be associated with decreased serum aB2GPI levels.

Our study has some limitations due to its retrospective design. Firstly, obesity that has been proposed to influence vitD levels in APS patients [12] was not recorded. Secondly, data on cardiovascular risk factors were missing in the control population. Thirdly, we could not analyze the effect of treatments, as they were considered as ever treatment, with no data about if they were prior or posterior to the determination of vitD serum levels. Finally, few studies were included in the metaanalysis and the thresholds for vitD insufficiency and/or deficiency were not homogeneous.

However, the high number of patients included in the study, the homogeneity of our population, the sex and age comparable control group, as well as the immunological profile and the statistical adjustments performed in the meta-analysis support our results.

In summary, our study suggests that APS patients have significantly lower serum vitamin D levels and higher frequency of vitamin D deficiency than healthy controls. Further studies are needed to assess the association between vitD and thrombosis in APS.

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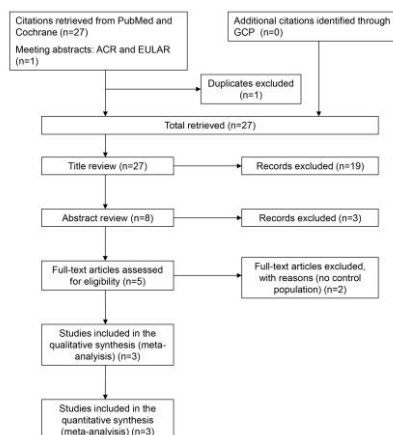
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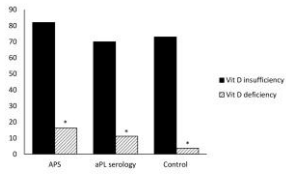
FIGURE LEGENDS

Figure 1. Flow diagram of the study selection process.

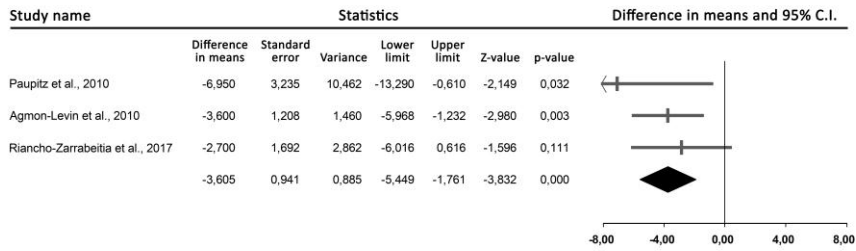
Figure 2. Frequencies of vitamin D insufficiency and deficiency in APS, patients with positive serology and controls. * $p=0.001$

Figure 3. Forest plot showing a) Differences in serum vitD levels between APS and controls. b) Differences in the frequency of vitD deficiency between APS and controls.

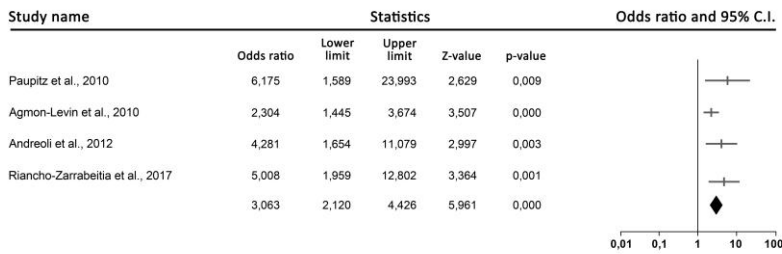




A



B



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