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# Evaluation of Adenovirus-36 (Ad-36) antibody seropositivity and adipokine levels in obese children

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ABSTRACT

Adenovirus 36 (Ad-36) has recently been suggested as a possible contributor to the current obesity epidemic. The aim of this study was to investigate the prevalence of Ad-36 antibodies in obese children, as well as investigate the role of serum leptin and lipid levels in Ad-36-obesity. Seventy-one obese children and 62 non-obese children were included as the patient group (PG), including the healthy control group (HCG), respectively. Simultaneously, Ad-36 antibodies and adipokine levels were assessed with serum neutralization assays (SNA) and ELISA. Ad-36 antibody was detected in 9 patients (12.7%) and 1 patient (1.6%) in both the PG and HCG, respectively, while a significant difference was detected between groups ($p < 0.05$). Although serum LDL, total cholesterol, triglycerides and leptin levels were detected significantly higher, adiponectin level was detected paradoxically lower in the PG. However, a significant difference was not detected for lipids and leptin levels; adiponectin levels were found to be significantly lower in Ad-36 antibody-positive PG ($p < 0.05$).

In conclusion, we suggest there is an association between Ad-36 and obesity in children, including IL-6 levels increasing in obese children with Ad-36 seropositivity. Conversely, adiponectin levels in obese children with Ad-36 seropositivity were higher. As such, there is a need for studies to understand the mechanisms underlying this observation.



## 1. Introduction

Recently, obesity is considered as one of the major public health problems to become a worldwide epidemic. Obesity also increases the risk of hypertension, coronary heart disease, stroke, and some cancers that cause serious morbidity and mortality. Additionally, obesity is also considered to be a state of low-grade chronic inflammation [1,2].

The prevalence of obesity in children and adults have also rapidly increased in Turkey [3]. Although energy intake of foods exceeds energy expenditure, and excess energy accumulates in adipose tissue as fat, it is accepted as the fundamental cause of obesity. Recently, infectious agents have been considered as potential etiological agents in the progression of obesity and the term “infectobesity”, which has similarly been suggested [4,5].

Human adenovirus-36 (Ad-36), first isolated in Germany in 1978 from the feces of a girl with enteritis and diabetes mellitus, is the first human virus linked with obesity in both animals and humans [6]. Ad-36-induced adiposity in a chicken model was reported by Dhurandhar et al. [7] for the first time. In the following experimental animal model studies, it was shown that Ad-36 can lead to obesity progression by causing hyperplasia and hypertrophy in the adipocytes of mice, rats, and monkeys [5,7,8]. In addition, serological and molecular studies, including children and adults,

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indicated evidence for an association between Ad-36 antibodies and obesity [9–22]. The presence of Ad-36 antibodies was detected in many serological studies with the serum neutralization assay (SNA), which is the accepted gold standard test [9,11,14,15,22].

One feature of obesity is an excessive increase in adipocyte proliferation of adipose tissue. It is known that levels of leptin, adiponectin, and IL-6 secreted from adipocytes show a great deal of diversity in obese people [23]. It has been suggested that changes in adipokine (such as leptin and adiponectin levels) may play a role in the pathogenesis of diseases such as Type II diabetes, cardiovascular disorders, and metabolic syndrome that accompany obesity. In this study, we investigated the presence of Ad-36 neutralizing antibodies and serum levels of leptin, adiponectin, and IL-6 in order to evaluate the Ad-36-obesity association in obese children.

## 2. Materials and methods

### 2.1. Patient and control groups

This study was sectional, and conducted in a case-controlled, randomized manner between June 2014 and May 2015, showing serum samples from The Pediatric Endocrinology and Outpatient Polyclinics. We included a patient group and a healthy control group, with 71 obese children between the ages of 7 and 17 (34 girls, 37 boys, mean age: 12.28 ± 2.60) and 62 children with normal weight (32 girls, 30 boys, mean age: 12.47 ± 2.70). The control group was consisted of 62 non-obese children who were admitted to the pediatric outpatient clinic for a check-up or routine health screening (No systemic antibiotic use within 6 months, and no current, chronic or acute disorders and medical conditions were reported). The patient and healthy control groups were matched in terms of age and gender ($p > 0.05$) (Table 1). Weight and height varies greatly during the adolescent period. In order to detect obese children, we used body mass index (BMI) z-score, based on World Health Organization (WHO) standards [24].

### 2.2. Collection of serum specimens

Serum specimens collected from patient and healthy control groups were stored at −80 °C until the SNA and ELISA tests were performed. Routine biochemical tests were done in the Biochemistry Laboratory of the same department (Roche HITACHI Cobas c501, US).

#### 2.2.1. Serum neutralization assay

SNA is accepted as the gold standard test for detection of Ad-36 in the serum of patient and healthy control groups. SNA was performed using the constant virus-varying serum technique. In the virus titration assay, a commercially obtained Ad-36 ATCC-VR 1610 (Rockville, MD, USA) strain was used. The infectious virus titer was seen as the dilution which caused 50% positivity of the virus in the A549 cell line (human lung carcinoma cell line, ATCC-CCL-185), calculated by Spearman-Kärber method [25]. The serum neutralization assay was performed by Dhurandhar et al. [26]. Serum samples were incubated at 56 °C for one half hour to inactivate the samples, which were diluted from 1:4 to 1:512 in 96 wells and microtiter plates. Moreover, 100 μL of Ad-36 suspension was added to each well and incubated at 37 °C for one hour. After incubation, 100 μL of A549 cell dilution at $2 \times 10^4$ cells per well was added to each one. Positive (virus and cells present, but no serum) and negative (only cells present) were prepared for each plate. The presence of cytopathic effect (CPE) was evaluated after the plates were incubated at 37 °C in a 5% $CO_2$ environment for 8–10 days.

**Table 1**
Characteristics of patient group and healthy control group.

| Characteristic/Variable | Patient group (n = 71) | Control group (n = 62) |
|---|---|---|
| Age, (mean ± sd) | 12.28 ± 2.60 | 12.47 ± 2.70 |
| Gender (girl, boy) | G:34, B:37 | G:32, B:30 |
| BMI z-score | 2.55 ± 0.56 | 0.39 ± 2.12 |
| Ad-36 presence | 9/71 | 1/62 |

#### 2.2.2. ELISA determination of serum leptin, adiponectin, and IL-6 levels

For the quantitative analysis of the leptin (Human Leptin ELISA DRG Diagnostics, Marburg, GY, Catalog No: EIA2395), adiponectin (Adiponectin, Assaypro LLC, St. Charles, MO, US, Catalog No: EA2500-1), and IL-6 (Human IL-6 ELISA Bioscience, San Diego, CA, Catalog No: 88-7066) levels for a commercial Sandwich ELISA (Sandwich Enzyme-Linked Immune Sorbent Assay) kit were used, according to the manufacturer's guidelines.

### 2.3. Statistical analysis

Fischer's exact test was used to compare the frequency and percentages for both patient and healthy control groups, with non-parametric comparisons using the Mann-Whitney *U* test. Variables significantly associated with obese children were tested for independence, along with a multivariate forward stepwise regression analysis. The biostatistical analysis of the study results was performed with the SPSS 21.0 program (Licensed to Istanbul University), and *p*-values under 0.05 were accepted as significant.

## 3. Results

Ad-36 positivity was detected at significantly higher levels (9 cases) in obese cases vs non-obese cases (one case) (Table 1). The presence of Ad-36 antibody in the patient group and healthy controls was found to be statistically significant ($p = 0.02$, odds ratio (OR): 8.855, 95% (min-max), confidence interval (CI): 1.089–72.022). Other parameters; mean leptin (PG:14.73, HCG:5.49, p = 0.0001), IL-6 (PG:3.93, HCG:2.28, p = 0.0001), LDL (PG:101.4, HCG:82.76, p = 0.0001, total cholesterol (PG:170.2, HCG:146.3, p = 0.0001) and triglyceride levels (PG:99.21, HCG:79.44, p = 0.011) between the patient group and healthy control group were detected to be significantly higher than healthy control group. Mean adiponectin (PG:9.60, HCG:16.69, p = 0.0001) levels were detected to be significantly higher than patient group. On the other hand, mean HDL levels (PG:50.11, HCG:56.06, p = 0.145) between the groups were not detected to be significantly different. The only 15 year old male with Ad-36 seropositivity in non-obese group had adinopectin levels at 17.15 ng/mL which were higher than the mean adinopectin levels (4.87 ng/mL) in obese infected and obese non-infected children (adinopectin:10.29 ng/mL). On the other hand, adinopectin levels in these children were very similiar with the mean adinopectin levels in non-obese children (16.69 ng/mL).

Mean serum adiponectin and IL-6 levels differed significantly between the Ad-36 antibody-positive and antibody-negative patient group ($p < 0.0001$). While the mean serum adiponectin levels were detected at significantly lower levels, IL-6 was higher in the Ad-36 antibody-positive patient group than the Ad-36 antibody-negative patient group ($p < 0.05$) (Table 2). Multivariate and bivariate logistic regression tests were performed to assess Ad-36 as a risk factor with demographic, biochemical, and virological variables (e.g., gender, age, triglycerides, HDL, LDL, adiponectin, and IL-6 levels). We found that Ad-36 was determined as a risk factor in the development of obesity by bivariate analyses ($p = 0.02$, OR: 8.855); however, Ad-36 was not determined as a risk factor by

**Table 2**
Comparison of mean BMI, leptin, adiponectin, cholesterol and triglyceride according to the presence of Ad-36 antibodies in the patient group.

| The presence of Ad-36 Antibody | | | |
|---|---|---|---|
| | Ad-36 (+) (n = 9) | Ad-36 (−) (n = 62) | p |
| Age | 12.44 ± 3.24 | 12.26 ± 2.53 | p > 0.05 |
| BMI | 30.5 ± 4.79 | 30.47 ± 3.97 | p > 0.05 |
| Leptin (μg/mL) | 20.48 ± 13.96 | 13.90 ± 9.65 | p > 0.05 |
| Adiponectin (ng/mL) | 4.87 ± 1.88 | 10.29 ± 13.46 | p < 0.05 |
| LDL (mg/dL) | 105.81 ± 32.57 | 100.8 ± 26.41 | p > 0.05 |
| HDL (mg/dL) | 49.00 ± 10.15 | 50.27 ± 11.05 | p > 0.05 |
| Total Cholesterol (mg/dL) | 175.7 ± 35.1 | 169.4 ± 30.2 | p > 0.05 |
| Triglyceride (mg/dL) | 100.2 ± 56.47 | 99.06 ± 43.6 | p > 0.05 |
| IL-6 (pg/mL) | 13.64 ± 10.22 | 2.52 ± 1.58 | p < 0.05 |

BMI; Body Mass Index, LDL; Low Density Lipoprotein, HDL; High Density Lipoprotein, Ad-36; Adenovirus 36.

multivariate logistic regression analysis.

## 4. Discussion

The prevalence of childhood obesity has been increasing at an alarming rate; globally, the number of overweight children under age five was estimated to be over 42 million in 2013 [27].

In the development of obesity, inflammation with an altered immune response in adipose tissue, suggesting an association between them. Adipokine secretion levels, inflammation originating in fatty acids, endoplasmic reticulum stress, oxidative stress, and hypoxia on adipocyte function are thought to be involved in the development of obesity [28,29]. The role of Ad-36 is pointed out in most of the studies as a function of obesity development.

Ad-36-induced adipocyte differentiation was first presented in animal studies by Dhurandhar et al. [8,26]. In a mouse model, it was suggested that the induction mechanism of adipocyte differentiation is initiated by the E4 (ORF1) viral gene of Ad-36, which infects the host cell nucleus; this indicates that adipogenesis is accelerated by the progression of adipocyte proliferation and differentiation; ultimately, cellular signaling pathways are affected as well [22,29]. Adipose tissue functions, such as those in the endocrine organs, secrete various inflammatory and anti-inflammatory cytokines with adipokines, so it is was also suggested that proinflammatory cytokines such as MCP-I, TNF-α, IL-1, and IL-6 increase the reproductive fat pads and alter fat metabolism during the inflammatory process; this is initiated by type $M_1$ macrophages, induced and infiltrated to the adipocytes by an increase of leptin and MCP-I levels. Using a similar approach, Na et al. [30] demonstrated in their mice models at 90 days, MCP-1 and mRNA levels were twice that of the TNF-α mRNA level, and 6 times higher in Ad-36 infected mice. In addition, the weight and size of reproductive fat pads were significantly greater in Ad-36 infected mice than in controls. On the other hand, MCP-1 knockout mice were protected from Ad-36 induced inflammation and obesity [30].

Studies indicate that adipocytes preserve their number and size under optimal conditions, although as obesity progresses, levels of proinflammatory cytokines and adipokines increase by augmented adipocytes [30]. In line with this, immune cells and macrophages infiltrate the adipocytes, given that remodeling of adipose tissue develops due to angiogenesis.

However, recent results of “Ad-36-obesity” association studies appear to yield conflicting information, supporting this association is conducted with adult cases [9,12,13]. The first study with children was conducted by Atkinson et al. [14] to determine the prevalence of Ad-36 infection in obese Korean children (8-16-years-old); there is a correlation of infection with the BMI z-score and other obesity measures. Thirty percent of subjects were positive (n = 25) for Ad-36, while 70% were negative (n = 59) with significantly higher BMI z-scores found in infected vs uninfected children in the study. In another study with Korean school children by Na et al. [15], the prevalence of Ad-36 antibody was higher in obese children (28%) than non-obese children (13%). Alhoon-Hainovera et al. [16] investigated the prevalence of Ad-36 in 1179 Czech adolescents, and the Ad-36 positive antibody was found to be higher in overweight and obese children (40% and 28%, respectively) suggesting an association of Ad-36 antibodies with obesity. No relation to adiponectin levels was revealed. Almgren et al. [18] found that the Ad-36 positive antibody was associated with pediatric obesity, and severe obesity in females compared to lean and overweight/mildly obese individuals, with a 1.5- to 2-fold increase.

Cakmakliogullari et al. [20] found Ad-36 antibody positivity to be higher in the obese group (26.6%) than in the non-obese group (10%). No significant difference was detected for other serum lipids. In one limited study reported from Turkey by Karamese et al. [21], Ad-36 seropositivity was significantly higher in obese children, which is similar to all of the studies, even those including children; however, no statistical difference was reported between Ad-36 seropositive and seronegative children with respect to TNF-α, IL-6, and serum lipids.

One limitation of this study was incubating the plates at 37 °C for 8–10 days compared to 11 days in the presence of CPE in the serum neutralization assay and thus some positives may have been missed. This may have led to lower prevalence in Ad-36 infections. All the studies published prior to 2012 have utilized SNA for detection of antibodies against Ad-36. Since performing SNA is time consuming (approximately 2 weeks) and costly, to alleviate this problem, ELISA detecting antibodies against a recombinant Adv36 coat protein (the fiber protein) in serum were developed by Almgren et al. [18]. However, we detected Ad-36 antibodies in 12.7% and 1.6% of the obese and non-obese children, respectively. Ad-36 was identified as an important risk factor for the development of obesity, with an OR value of 8.855 by bivariate logistic regression analysis. Similarly, in the study by Cakmakliogullari et al. [20], Ad-36 was identified as a risk factor with an OR value of 1.859 in the development of obesity. In the study of Na [15], the multivariate logistic regression analysis indicated only Ad-36 positivity to be a significant risk factor, with an OR of 2.550. In this study, the leptin levels of the Ad-36 antibody-positive patient group were found to be higher, but no statistically significant difference was detected between these two groups. However, our leptin results are similar to the results of Cakmakliogullari et al. [20]. On the other hand, our leptin results do not correspoind to those of Karamese et al. [21].

The role of adipokine levels in Ad-36-obesity by Aldhoon-Hainerova et al. [16] found a statistically significant difference between obese and non-obese children with respect to adiponectin levels, in accordance with our own study. However, in comparison to our results, there was no relation of adiponectin levels with Ad-36. Serum adiponectin levels are generally low in obesity [31]. However, those naturally infected with Ad-36 were found to have significantly greater adiponectin levels, which may create better glycemic control, as some studies suggest that Ad-36 may increase adiponectin [32–34]. The only 15 year old male with Ad-36 seropositivity in non-obese group had adinopectin levels at 17.15 ng/mL which were higher than the mean adinopectin levels (4.87 ng/mL) in obese infected children and obese non-infected children (adinopectin:10.29 ng/mL). On the other hand, adinopectin levels in these children were very similiar with the mean adinopectin levels in non-obese children (16.69 ng/mL). The LDL (101 mg/dL), total cholesterol (168 mg/dL), and triglyceride levels of the same group were similiar and slightly higher than those of obese-infected children. Mamaghani et al. [35] showed that serum

adiponectin levels decreased with obesity and were accompanied by increases in serum leptin levels. They suggested that adiponectin had an inverse correlation with adiposity. Our findings showing decreased adinopectin and increased leptin levels in obese infected children are in agreement with the aforementioned report. The fifteen year old male child with Ad-36 seropositivity in non-obese group is also in agreement with the literature. Because this individual had increased adinopectin levels (17.15 ng/mL) and decreased leptin levels (0.9 ng/mL), we suggest that Ad-36 facilitates the development of obesity; however, we couldn't detect the causal effect of Ad-36 for adipogenesis in this 15 year old. Although our study was conducted with children, the adiponectin results of a previous study by Ergin et al. [13] with adults were similar to our own results for the pediatric group.

It has been suggested that IL-6 levels increase with obesity in a single study by Karamese et al. [21], but no statistically significant difference was identified between the IL-6 levels of Ad-36 seropositive and seronegative children. Our findings relate to detecting higher IL-6 levels in obese children with Ad-36 seropositivity, which is in agreement with the literature shown below. Berger et al. [36] reported that the prevalence of Ad-36 to be significantly associated with higher IL-6 levels in 122 (Ad-36 seropositive) of 291 obese children aged 9–13 years ($p = 0.03$). It was also reported that interleukin 6 (IL-6) expression in adipocytes in vitro was increased by Ad-36 infection, which may help with the maintenance of a chronic low-grade inflammation state [37]. As is known, inflammation also contributes to the maintenance of the obesity state [38]. We detected IL-6 levels of Ad-36 antibody-seropositive children significantly higher than seronegative children [21]. However, despite this result, the IL-6 level was not identified as a risk factor by multivariate logistic analysis in our study.

In conclusion, we find an association between Ad-36 and obesity in children; moreover, IL-6 levels were increased in obese children with Ad-36 seropositivity. On the other hand, adiponectin levels in obese children with Ad-36 seropositivity were lower than seronegative ones; thus, there is a need for comprehensive studies to better understand these mechanisms. However, the role of serum lipids and adipokines still seem inconsistent for this association, including how the parameters with Ad-36 are not clear (especially if the link between microorganisms such as Ad-36 and obesity demonstrates that it will become easier to develop strategies for treatment, prevention, and vaccination for it).

## Ethics

This study was approved by the Clinical Research Ethics Committee of Istanbul University, Cerrahpasa Medical Faculty (Number: 114279, 04 July 2014). The parents of all children included in this study were briefed about the methodology, and signed written consent forms.

## Conflict of interest

The authors report no conflicts of interest relevant to this article.

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## Author contributions

HY, BK and SS designed the research; PC, NI, SS, OE and BTO were responsible for clinical specimen collection; performed the statistical analyses; SE and OD wrote the manuscript; EAT, ZT, AK, KA, PE, NT, UCand SS was responsible for laboratory analyses; all authors contributed to the critical revision of the manuscript.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micpath.2017.04.034.

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