

## Original Article

# Genetic Variation in *NFKB1* Gene Influences Liver Enzyme Levels in Morbidly Obese Women

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## Abstract

**Background:** Morbid obesity (MO), characterized by low-grade inflammation, is associated with increased C-reactive protein (CRP). NF- $\kappa$ B is a candidate factor for inflammatory responses in inflammatory diseases such as obesity. The objective of our study was to investigate the relationship between *NFKB1* gene variations and the risk of MO in the context of the high/normal level of liver enzymes such as Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), and alkaline phosphatase (ALP).

**Methods:** We analyzed the distribution of *NFKB1* -94 ins/del ATTG (rs28362491) polymorphism using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and liver enzymes serum levels using ELISA in 182 MO patients with CRP level  $\geq 20$  mg/L and 200 healthy controls in a female Turkish population.

**Results:** We found that having ins/ins genotype of rs28362491 is a risk factor in both high level and normal level liver enzymes of ALT ( $P = 0.0335$ ,  $P = 0.0134$ ), AST ( $P = 0.0285$ ,  $P = 0.0113$ ) and ALP ( $P = 0.0079$ ,  $P = 0.0363$ ) whereas having ins/ins genotype of rs28362491 is a risk factor in only high-level liver enzyme of GGT ( $P = 0.0003$ ).

**Conclusion:** Our results suggest that ins/ins genotype of SNP rs28362491 is linked to MO with high-level ALT, AST, ALP, and GGT.

**Keywords:** Liver enzymes, Morbid obesity, *NFKB1*, Polymorphism

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## Introduction

Obesity, a major health problem worldwide, is a multifactorial disorder arising from the interplay between genetic susceptibility and environmental influences.<sup>1</sup> Morbid obesity (MO), the most severe form of obesity, is defined as body mass index (BMI)  $> 40$  kg/m<sup>2</sup> and mainly characterized by low-grade inflammation in both blood and white adipose tissue (WAT) that is associated with increased C-reactive protein (CRP), a well-known acute phase protein. CRP is a marker frequently measured in clinical practice to evaluate systemic inflammation, especially in cases of infection.<sup>2</sup> Studies have reported positive correlations between CRP and BMI as reviewed by Choi et al.<sup>3</sup>

Part of the systemic inflammation associated with obesity in which many inflammatory cells tend to accumulate, originates from WAT itself.<sup>4,5</sup> Moreover, fat accumulation in the liver can also stimulate cytokine production and it has been found that the inflammatory cytokines such as interleukin 6 (IL-6), Tumor necrosis

factor alpha (TNF- $\alpha$ ) can influence fatty acid metabolism in the liver.<sup>6</sup> On the other hand, about 90% of morbidly obese patients show histological abnormalities of the liver. Visceral fat accumulation is particularly dangerous. It leads to metabolic syndrome and is associated with type 1 T helper cell (Th1)-weighted chronic, low-grade systemic inflammation. The accumulation of fat alone is unlikely to be the stimulus for inflammation.<sup>7</sup>

A number of genes have been identified as linked to the onset of obesity or an increase in the susceptibility to this disorder. Given its place as a master regulator at the center of inflammation, nuclear factor- $\kappa$ B (NF- $\kappa$ B) is a natural suspect in providing a mechanistic link between inflammation and MO.<sup>8</sup> The human NF- $\kappa$ B family consists of 5 cellular DNA-binding subunits: p50, p52, cRel, p65 (also called RelA) and RelB, encoded by *NFKB1*, *NFKB2*, *REL*, *RELA*, and *RELB*, respectively.<sup>9</sup> It is known that *NFKB1* encodes the p50/p105 subunits and deletion of four nucleotides in the promoter region (-94ATTG) of *NFKB1* gene (rs28362491) results in

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lowered transcription levels partial deletion. Recently, we reported that *NFKB1* promoter polymorphism is associated with susceptibility to MO through acting by modulating serum CRP levels.<sup>10</sup> As reported previously, obesity increases active, nuclear-localized NF-KB in the liver and skeletal muscle and transcription of NF-KB target genes.<sup>11</sup>

The role of hepatic enzymes as markers of the fatty liver disease has been detected in obesity. Alkaline phosphatase (ALP) and gamma-glutamyl transpeptidase (GGT) are sensitive markers for liver damage. GGT, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are correlated with BMI; GGT and ALP are significantly higher in obese subjects; ALT and AST are markers of hepatocyte injury and liver fat accumulation and GGT is an indicator of biliary and heavy alcohol consumption.<sup>12-14</sup>

Finding the correlation with gene polymorphisms, high-level liver enzymes in the case of inflammation might help to elucidate the genetic characteristics of individuals who are susceptible to liver dysfunction and related conditions such as metabolic syndrome or fatty liver disease. No research has been conducted on the polymorphism of *NFKB1*, liver enzymes and MO in a Turkish population. In this context, we hypothesize that MO would expose the association between the *NFKB1* rs28362491 variant and liver parameters.

## Patients and Methods

### Subjects

The study was approved by the Institutional Ethical Committee of Bezmialem University (Project No: 6.2015/7 (2015)). All participants gave written informed consent for participation in this medical research. We included 182 morbidly obese (MO) women (mean age:  $38.0 \pm 5.7$  years) and 200 healthy women (mean age:  $39.0 \pm 7.3$ ) of Turkish origin. Obesity was diagnosed according to BMI. MO women with BMI  $>35$  kg/m<sup>2</sup> and CRP level  $\geq 20$  mg/L and healthy women with BMI  $<25$  kg/m<sup>2</sup> were included in the study. We excluded normal-BMI women who had any systemic disease including liver, chronic kidney or cardiovascular diseases, or malignancies, rheumatologic disorders or any inflammatory diseases as well as subjects in both normal and obese groups who used alcohol.

### Blood Samples and DNA Isolation

The blood samples were collected in plastic vacutainer tubes containing EDTA for DNA isolation and without additives for serum CRP and liver enzymes level. Immediately after collection, serum CRP and liver enzyme levels were measured by ELISA. Whole blood samples were stored at  $-20^{\circ}\text{C}$  for genomic DNA extraction.

Genomic DNA was extracted from blood using Roche DNA purification kit (Roche Diagnostics GmbH, Mannheim, Germany; Product No. 11796828001) according to the manufacturer's instructions. The concentration and purity of DNA samples were established using a NanoDrop<sup>TM</sup> spectrophotometer. DNA samples with OD ratio  $1.8 \pm 0.1$  were included in the study.

### Polymorphism Analysis

The polymorphisms of *NFKB1* gene were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The 285/281 base-pair PCR fragment of the *NFKB1* gene was amplified in a 25  $\mu\text{L}$  reaction volume containing 100 ng genomic DNA, 200 pmol of each dNTPs, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1 U Taq Polymerase (Sigma, St. Louis, MO, USA) and 2 mM MgCl<sub>2</sub> (Fermentas, Lithuania). Primers were F:5'- TGGGCACAAGTCGTTTATGA-3' and R:5'- CTGGAGCCGGTAGGGAAG-3'. PCR conditions were as follows: 95°C for 1 minute; 35 cycles of 95°C for 30 seconds, 61°C for 30 seconds, 72°C for 1 minute; and a final incubation at 72°C for 5 minutes. On completion of PCR, the products were digested overnight with 5 U of PflMI (Van91I) (Fermentas, Lithuania) at 37°C and run on an ethidium bromide-stained 3% agarose gel for 45 minutes at 90 V and finally screened under UV light to detect two different alleles of rs28362491, the 281 bp (deletion-wild type allele) or 285 bp (insertion allele). As deletion genotype has no PflMI (Van91I) restriction site, the PCR product of 281 bp remained undigested. On the other hand, the insertion variants were cleaved by PflMI (Van91I) restriction enzyme into two fragments of 240 bp and 45 bp. Heterozygotes showed all three bands.

### Statistical Analysis

GraphPad Prism 5 program was used for data analysis of the patients and controls values. Genotype and allele frequencies were compared between the cases and the controls by chi-square analysis. Odds ratio (OR) and respective 95% confidence intervals (CIs) were reported to evaluate the effects of any difference between allelic and genotype distribution. A two-sided *P* value  $\leq 0.05$  was considered statistically significant. The enzyme level data were expressed as a mean  $\pm$  standard deviation (SD). Student's *t* test was used for comparison between these groups.

## Results

In the case of ALT, ALP, AST and GGT enzymes, a significant relation ( $P < 0.05$ ) was found when control and patient groups were compared (See Table 1).

Table 2 presents the distribution of the allele and

**Table 1.** Values of Liver Enzymes ALT,ALP,AST and GGT (in U/L) in Healthy Control and Patient Groups

	Control (n), (Mean ± SD)	Patient (n), (Mean ± SD)	P Value
ALT	(n=182), (18.27 ± 8.31)	(n=182), (37.56 ± 24.47)	<b>&lt;0.0001</b>
ALP	(n=182), (64.55 ± 18.44)	(n=179), (79.39 ± 22.62)	<b>&lt;0.0001</b>
AST	(n=182), (24.50 ± 23.58)	(n=182), (28.98 ± 15.30)	<b>0.0322</b>
GGT	(n=182), (18.53 ± 8.78)	(n=176), (31.73 ± 18.58)	<b>&lt;0.0001</b>

Abbreviations: ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase.

genotype frequencies of the *NFKB1* rs28362491 in control group and MO patients who were divided into 2 groups: high levels of serum ALT (HL ALT ≤40 U/L) and normal levels of serum ALT (NL ALT >40 U/L).

According to Table 2, having ins/ins genotype of rs28362491 has a 2.06-fold risk in MO group with HL ALT (95% CI:1.119–3.864, *P* = 0.0335) and 1.83-fold risk in MO group with NL ALT (95% CI: 1.134–2.98, *P*=0.0134). Regarding allele frequency, ins allele of rs28362491 was determined to be a risk factor in MO group with NL ALT (OR: 1.448, 95% CI: 1.04–2.015, *P*=0.0303). There were no significant differences in the distribution of rs28362491 allele frequencies between patients and the healthy control group in the case of HL ALT (*P* > 0.05).

Table 3 indicates the distribution of *NFKB1*

rs28362491 allele and genotype frequencies in control group and MO patients who were divided into two groups: high levels of serum ALP (HL ALP ≤80 U/L) and normal levels of serum ALP (NL ALP >80 U/L).

According to our results, having ins/ins genotype of rs28362491 has a 2.044-fold risk in MO group with HL ALP (95% CI: 1.182–3.45, *P* = 0.0079) and 1.766-fold risk in MO group with NL ALP (95% CI: 1.048 – 2.988, *P* = 0.0363). Regarding allele frequency, ins allele of rs28362491 was determined to be a risk factor in MO group with HL ALP (OR: 1.599, 95% CI: 1.092–2.321, *P* = 0.0151). In addition, there were no significant differences in the distribution of rs28362491 allele frequencies between patients and the control group in the case of NL ALP (*P* > 0.05) (See Table 3).

Table 4 presents the distribution of *NFKB1* rs28362491 allele and genotype frequencies in control group and MO patients who were divided into two groups: high levels of serum AST (HL AST ≤30 U/L) and normal levels of serum AST (NL AST > 30 U/L).

According to our results, having ins/ins genotype of rs28362491 has a 1.969-fold risk in MO group with HL AST (95% CI: 1.144–3.577, *P* = 0.0285) and 1.866-fold risk in MO group with NL AST (95% CI: 1.147 – 3.064, *P* = 0.0113). In addition, regarding allele frequency, ins allele of rs28362491 was found to be a risk factor in

**Table 2.** Alanine Aminotransferase Serum Levels of *NFKB1* -94ins/del Polymorphism in Morbid Obesity Subgroups and Healthy Control

Genotype/Allele	Control, No. (%)	Morbid Obesity Subgroups					
		ALT >40 Group, No. (%)	<i>P</i>	OR (95% CI)	ALT <40 Group, No. (%)	<i>P</i>	OR (95% CI)
<b>NFKB</b>							
ins/ins	64 (32)	28 (47.5)	-	Ref.	57 (46.3)	-	Ref.
del/ins	113 (56.5)	24 (40.7)	<b>0.0335</b>	2.06 (1.119–3.864)	55 (44.7)	<b>0.0134</b>	1.83 (1.134–2.98)
del/del	23 (11.5)	7 (11.9)	0.6070	1.438 (0.5561–3.631)	11 (9)	0.7937	0.1815 (0.8492–3.98)
ins/ins+ del/ins / del/del	-	-	0.8771	0.9653 (0.3905–2.562)	-	0.5889	1.323 (0.6201–2.912)
ins/ins /del/ins +del/del	-	-	0.0292	1.919 (1.051–3.477)	-	0.0097	1.835 (1.144–2.874)
ins allele frequency	241(60)	80 (68)	-	Ref.	169 (68.7)	-	Ref.
del allele frequency	159 (40)	38 (32)	0.1378	0.1378 (0.9071–2.165)	77 (31.3)	<b>0.0303</b>	1.448 (1.04–2.015)

Abbreviation: ALT, alanine aminotransferase.

**Table 3.** Alkaline Phosphatase Serum Levels of *NFKB1* -94ins/del Polymorphism in Morbid Obesity Subgroups and Healthy Control

Genotype/Allele	Control, No. (%)	Morbid Obesity Subgroups					
		ALP ≥80 Group, No. (%)	<i>P</i>	OR (95% CI)	ALP <80 Group, No. (%)	<i>P</i>	OR (95% CI)
<b>NFKB</b>							
ins/ins	64 (32)	44 (49.4)	-	Ref.	40(44)	-	Ref.
del/ins	113 (56.5)	38 (42.7)	<b>0.0079</b>	2.044 (1.182–3.45)	40(44)	<b>0.0363</b>	1.766 (1.048–2.988)
del/del	23 (11.5)	7 (7.9)	0.1251	2.259 (0.5561–3.631)	11(12)	0.7937	0.6629 (0.5732–2.827)
ins/ins+ del/ins / del/del	-	-	0.4676	1.522 (0.6379–3.936)	-	0.9585	0.945 (0.4345–2.115)
ins/ins /del/ins +del/del	-	-	<b>0.0047</b>	2.078 (1.248–3.476)	-	<b>0.0485</b>	1.667 (0.9974–2.765)
ins allele frequency	241(60)	126 (70.8)	-	Ref.	120(66)	-	Ref.
del allele frequency	159 (40)	52 (29.2)	<b>0.0151</b>	1.599 (1.092–2.321)	62(34)	0.1902	1.277 (0.8922–1.829)

Abbreviation: ALP, alkaline phosphatase.

MO group with NL AST (OR: 1.533, 95% CI: 1.087 – 2.168,  $P = 0.0143$ ). However, there were no significant differences in the distribution of rs28362491 allele frequencies between patients and the control group in the case of HL AST ( $P > 0.05$ ) (See Table 4).

Table 5 indicates the distribution of *NFKB1* rs28362491 allele and genotype frequencies in control group and MO patients who were divided into 2 groups: high levels of serum GGT (HL GGT  $\leq 37$  U/L) and normal levels of serum GGT (NL GGT  $> 37$  U/L).

According to our results, having ins/ins genotype of rs28362491 has a 3.649-fold risk in MO group with HL GGT (95% CI: 1.817–7.191,  $P = 0.0003$ ). Regarding allele frequency, ins allele of rs28362491 was found to be a risk factor in MO group with NL GGT (OR: 1.533, 95% CI: 1.087–2.168,  $P = 0.0143$ ). There were no significant differences in the distribution of rs28362491 allele frequencies between patients and the control group in terms of both NL GGT and HL GGT (See Table 5).

## Discussion

The most serious form of obesity, MO, is a multifactorial disease and associated with inflammation.<sup>1</sup> The distribution of CRP, a marker of systemic inflammation in MO, is considered as a model of extreme adipose

tissue expansion. In obese patients, presenting with high CRP levels above 20 mg/L would be related to fat mass expansion and adipose tissue inflammation. Moreover, the authors in one report excluded patients with CRP  $>20$  mg/L considering that an infection or a systemic inflammatory disorder other than obesity could exist.<sup>15</sup> In this context, we preferred to choose MO patients with CRP  $>20$  mg/L assuming that these patients have inflammation.

There is a good evidence of an association between hepatic fat accumulation and chronic inflammation; a key player appears to be the NF-KB gene that encodes the p50/p105 subunits. The deletion *NFKB1*-94ins/delATTG (rs28362491) promoter region leads to lower levels of the p50 subunit, and this affects both the availability of the anti-inflammatory p50/p50 NF-KB homodimer and the pro-inflammatory p50/p65 NF-KB heterodimer. Consistent with these findings, we have reported the association of this polymorphism of *NFKB1* gene with many inflammatory diseases such as Hashimoto thyroiditis,<sup>16</sup> Graves' disease<sup>17</sup> and Behcet's disease.<sup>18</sup> CRP transcription is controlled by the p50 homodimer of NF-KB.<sup>19</sup> We have previously found higher levels of CRP protein in ins-allele carriers compared to del-allele carriers,<sup>10</sup> supporting the interpretation that the del-allele

**Table 4.** Aspartate Aminotransferase Serum Levels of *NFKB1* -94ins/del Polymorphism in Morbid Obesity Subgroups and Healthy Control

Genotype/Allele	Control, No. (%)	Morbid Obesity Subgroups					
		AST $\geq 30$ Group, No. (%)	<i>P</i>	OR (95% CI)	AST $< 30$ Group, No. (%)	<i>P</i>	OR (95% CI)
<b>NFKB</b>							
ins/ins	64 (32)	29 (45.3)	-	Ref.	56 (47.4)	-	Ref.
del/ins	113 (56.5)	26 (40.6)	<b>0.0285</b>	1.969 (1.044–3.577)	53 (45)	<b>0.0113</b>	1.866 (1.147–3.064)
del/del	23 (11.5)	9(14.1)	0.9191	1.158 (0.4934–2.717)	9 (7.6)	0.0924	2.236 (0.9524–5.486)
ins/ins+ del/ins / del/del	-	-	0.7439	0.7941 (0.3554–1.896)	-	0.3596	1.574 (0.7363–3.618)
ins/ins /del/ins +del/del	-	-	0.0523	1.761 (0.9941–3.075)	-	0.0060	1.919 (1.212–3.039)
ins allele frequency	241(60)	84(65.6)	-	Ref.	165 (70)	1.533	Ref
del allele frequency	159 (40)	44 (34.4)	0.2766	1.26 (0.8317–1.926)	71 (30)	<b>0.0143</b>	1.533 (1.087–2.168)

Abbreviation: AST, aspartate aminotransferase.

**Table 5.** Gamma-glutamyl transpeptidase Serum Levels of *NFKB1* -94ins/del Polymorphism in Morbid Obesity Subgroups and Healthy Control

Genotype/Allele	Control, No. (%)	Morbid Obesity Subgroups					
		GGT $> 37$ Group, No. (%)	<i>P</i>	OR (95% CI)	GGT $\leq 37$ Group, No. (%)	<i>P</i>	OR (95% CI)
<b>NFKB</b>							
ins/ins	64 (32)	31 (56.3)	-	Ref.	53 (42.1)	-	Ref.
del/ins	113 (56.5)	15 (27.3)	<b>0.0003</b>	3.649 (1.817–7.191)	64 (50.8)	0.1170	1.462 (0.9113–2.348)
del/del	23 (11.5)	9 (16.4)	0.7990	1.238 (0.5333–2.905)	9 (7.1)	0.1225	2.116 (0.8967–5.117)
ins/ins+ del/ins / del/del	-	-	0.4627	0.6642 (0.2918–1.609)	-	0.2729	1.689 (0.7893–3.873)
ins/ins /del/ins +del/del	-	-	<b>0.0016</b>	2.745 (1.472–5.013)	-	0.0651	1.543 (0.9597–2.477)
ins allele frequency	241 (60)	77 (70)	-	Ref.	170 (67.5)	-	Ref.
del allele frequency	159 (40)	33 (30)	0.0616	1.539 (0.9725–2.442)	82 (32.5)	0.0633	1.368 (0.9769–1.912)

Abbreviation: GGT, gamma-glutamyl transpeptidase .



causes depletion of the p50 homodimer.

In the present study, the effect of *NFKB1* ins/del ATTG polymorphism and the elevation of serum liver enzymes, namely AST, ALT, ALP, and GGT were observed in healthy control group and the MO group with high CRP (> 20 mg/L) and we showed that ALT, AST, ALP and GGT serum levels significantly increased in patients with the ins/ins genotype.

We also estimated the values of liver enzymes (ALT, AST, ALP, GGT) of individuals in case of *NFKB1* genotypes according to having high levels (HL) and normal levels (NL) of the liver enzyme. The results were similar for all 4 enzymes. In the case of ALT, ALP and AST levels, having ins/ins genotype of rs28362491 led to a risk in both HL and NL groups, interestingly higher in HL enzyme. As summarized in Table 5, in the case of GGT levels, instead, having ins/ins genotype of rs28362491 led to a risk in only MO group having HL GGT. In addition, the frequency of ins allele of rs28362491 was found to have a high risk in MO groups having NL ALT, HL ALP, and NL AST.

It is known that ALT, ASP, and GGT are markers of hepatocyte injury and liver fat accumulation.<sup>13,14,20</sup> Fatty liver is associated with elevated serum ALT and GGT concentrations and these commonly measured factors are now considered surrogate markers of liver fat accumulations. Both ALT and GGT appear to correlate with the amount of liver fat presence measured by MRI.<sup>21,22</sup> Ali Ibrahim Al-Sultan in his major study has pointed out that ALT, AST, ALP, and GGT are linked with obesity in Saudi Arabia.<sup>23</sup> In another study, Marchesini et al concluded that AST and GGT levels in obesity are associated with insulin resistance and metabolic syndrome.<sup>24</sup>

The literature has many conflicting results. In one report, the elevation in liver enzymes especially high serum ALT activity was associated with obesity and even within the normal range, ALT correlated with increased liver fat.<sup>25</sup> Contradicting with this result, high levels of GGT and ALP were shown to be significant parameters whereas ALT and AST were not different between non-obese and obese subjects.<sup>25-27</sup>

In research on obese patients by analyzing ALT, AST and GGT, Marchesini et al<sup>24</sup> reported not only that median ALT & AST increased with increasing obesity class ( $P = 0.001$  and  $P = 0.005$ ) and exceeded normal limits in 21.0% of cases but also HOMA-IR increased with the obesity class ( $P < 0.0001$ ) and was higher in subjects with elevated ALT ( $P < 0.0001$ ).

In one well-known recent experiment, Strauss et al<sup>28</sup> showed that 60% of adolescents with elevated ALT levels were either overweight or obese, and approximately 1% of obese adolescents demonstrated ALT levels over

twice normal.

Surveys such as that conducted by Choi have shown that mean activity ( $\pm$  SD) of serum ALT and AST in men with high fatness was significantly higher than those in men with low fatness ( $P < 0.01$ ). Out of 147 men with high fatness, they found that 56 (38.1%) had serum ALT levels above the upper limit of normal, whereas only 9.5% (31/328) of men with low or desirable fatness showed elevated serum ALT activities ( $P < 0.01$ ) and concluded that serum ALT, AST & GGT activities correlated significantly with total body fat (TBF) in both overweight men and women.<sup>29</sup>

The results of the current study support that levels of ALT, AST, ALP, and GGT were significantly increased in MO patients compared to control groups suggesting that liver enzymes levels are probable biomarkers in MO development.

Taken together, these results suggest an association between ins/ins genotype of SNP rs28362491 and MO in the context of the high level of ALT, ALP, AST and GGT enzymes. *NFKB1* gene might be helpful for monitoring the course and severity of liver disease and evoke new ways of therapies for these conditions.

#### Authors' Contribution

In the project, GY, TS and HA were responsible for laboratory tasks; ET was the clinician; GK was the advisor; TS and HA were also responsible for statistical calculations. GY and GK wrote the manuscript.

#### Conflict of Interest Disclosures

The authors have no conflicts of interest.

#### Ethical Statement

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Study protocol was approved by Institutional Ethical Committee of Bezmialem University with decision number of 03/06/2015 - 11/10.

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