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The A allele of cluster of differentiation 36 (*CD36*) SNP 1761667 associates with decreased lipid taste perception in obese Tunisian women

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Abstract

Recent studies have suggested that excessive intake of dietary fat is associated with obesity. Some obese subjects have been reported to exhibit high thresholds for the gustatory detection of lipids via lipid receptors, such as cluster of differentiation 36 (CD36). We studied lingual detection thresholds for emulsions containing oleic acid in obese Tunisian women (*n* 203) using a three-alternative forced choice (3-AFC) method. Genotyping of the *TNF-α* (rs1800629), *IL-6* (rs1800795) and *CD36* (rs1761667) genes was performed to associate with lipid taste perception thresholds. The *CD36* genotype distribution was as follows: GG (*n* 42), AG (*n* 102) and AA (*n* 59). Women with the *CD36* GG genotype exhibited oral detection thresholds for oleic acid that were more than three times lower than those with the *CD36* AA genotype. The present study confirms a high threshold of gustatory fat detection in obese women with the *CD36* AA genotype, but there is no significant association with the *IL-6* and *TNF-α* gene polymorphisms.

Key words: **Obesity: Lipids: Taste: Genes**

As the obesity epidemic continues, more subjects are getting fatter and are therefore at increased risk for metabolic complications, hypertension and cancer-related mortality[1,2]. The aetiology of obesity is multifactorial, and genetic inheritance and behavioural/environmental causes are considered to be the main factors[3]. Dietary fat is considered palatable to humans, and several factors, including its olfactory, visual and textural properties, have been proposed as playing a key role in the attractiveness of fat[4]. Humans and rodents can detect long-chain fatty acids in their diets as gustatory cues[4–9]. Some recent studies have shown that obese subjects exhibit a high preference for dietary lipids as compared to lean subjects[10,11], which suggests that inappropriate lipid perception might influence obesity risk by impacting feeding behaviour. In fact, obesity is associated with a low sensitivity to the oro-sensorial detection of fat[10,11].

Lingual cluster of differentiation 36 (CD36), like G protein-coupled receptor 120 (GPR120) and G protein-coupled receptor 40 (GPR40), has been shown to act as a lipid receptor that is involved in a spontaneous preference for fat[6,12–15]. The lingual lipid receptors bind to long-chain fatty acids, which are released by lingual lipases in the buccal cavity[12–15]. We performed the present study on *CD36* SNP because CD36 is a high-affinity receptor, whereas GPR120 and GPR40 are low-affinity receptors. In addition, GPR40 could not be detected on human lingual epithelium[16]. Moreover, Sclafani *et al.*[17] have shown that CD36 is directly involved in early fat detection, whereas GPR120 plays a role in the post-ingestive regulation of fat preference[18].

Recent studies have shown that CD36 protein expression is influenced by *CD36* gene polymorphism, and it is related to the detection threshold of dietary lipids in obese subjects[19]. Keller *et al.*[20] reported that obese subjects with the *CD36* AA genotype (rs1761667) perceived more creaminess in salad as compared to those with the AG or GG genotypes. Pepino *et al.*[19] reported that obese subjects with the AA genotype exhibited higher oral detection thresholds for fat than those with the AG and GG genotypes. These novel findings

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Abbreviations: GPR120, G protein-coupled receptor 120; HbA1c, glycosylated Hb.

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are changing our view about the pathogenesis of obesity; however, future studies must be conducted to confirm these interesting findings, particularly in developing countries where obesity is quickly rising.

In chronic pathological conditions such as obesity, IL-6 plays a synergic role in inflammation[21–23], because macrophages within adipose tissue might secrete IL-6[22]. An association between the rs1800795 polymorphism of the *IL-6* gene and increased adiposity, inflammation and metabolic disturbances has been demonstrated[24,25]. In obesity, adipose tissues also secrete TNF-α abundantly[26], and rs1800629 polymorphism of the *TNF-α* gene[27] has been reported to be associated with obesity risk[28] as well as a high incidence of type 2 diabetes[29]. Because obesity is marked by inflammation, the present study is also designed to explore the relationship between pro-inflammatory markers (IL-6, TNF-α and C-reactive protein) and the oro-sensorial detection of lipids in obese subjects.

There has recently been a rapid upsurge in overweight/obesity and obesity-related diseases in Tunisia, especially in women as compared to men[30,31]. This sex gap between women and man differs greatly according to environmental and socio-economic conditions[31]. Keeping in mind the aforementioned literature on lipid oral taste sensitivity and *CD36* SNP, we thought it would be worthwhile to investigate whether *CD36* SNP in obese Tunisian women is associated with decreased fat taste perception.

Materials and methods

Subjects

Inclusion criteria. Obese women (*n* 203) were recruited from the group of patients who visited the gynaecology outpatient department (OPD) of Farhat Hached University Hospital, Sousse (Tunisia), in 2012 and 2013 for a general health check-up. Medical records were screened by specialist clinicians. The studied women were between 38 and 43 years old. The women were asked to return to the gynaecology OPD when they were in their first week of menstruation, and they were given an appointment for a particular date so that blood sampling and an analysis of other parameters could be performed.

The exclusion criteria included smoking, diabetes, breastfeeding, pregnancy-related complications, a history of gestational diabetes, the use of oral contraception, chronic illness such as hypertension or any other inflammatory pathology, any autoimmune disease, any lipid-lowering medication, recent weight loss, dieting and the use of any medications known to affect taste. The inclusion criterion constituted a normal glucose tolerance test and electrocardiograms.

Anthropometrics. Body weight and height were measured in the morning while participants were unclothed and not wearing shoes. BMI was calculated as body weight (in kg) divided by height (in m^2). Obesity was defined as a BMI of 30 kg/m^2 or higher, in accordance with the recommendations of WHO. The characteristics of the women are shown in Table 1.

Table 1. Clinical characteristics of obese Tunisian women (*n* 203)
(Mean values and standard deviations)

Parameters	Mean	SD
Weight (kg)	86·4	16·3
Height (cm)	158	6
BMI (kg/m^2)	34·6	4·2
TNF-α (pg/ml)	131·4	165·5
IL-6 (pg/ml)	73·3	48·7
Age (years)	38·4	11·4
Glucose (mmol/l)	5·97	1·84
Urea (mmol/l)	3·39	1·58
Creatinine (μmol/l)	60·39	14·58
Cholesterol (mmol/l)	5·08	1·29
TAG (mmol/l)	1·77	1·06
HDL (mmol/l)	1·14	0·31
LDL (mmol/l)	3·04	1·01
HbA1c (%)	5·60	1·22
ApoA1 (g/l)	2·04	0·74
ApoB (g/l)	1·26	1·13
Insulin (pmol/l)	78·76	67·64
CRP (mg/l)	7·8	7·8

HbA1c, glycosylated Hb; CRP, C-reactive protein.

Ethics

The present study was carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association and was approved by Farhat Hached Hospital Committee for Research on Human Subjects (Tunisia). Informed written consent was obtained from all of the subjects. The present experimental protocol conforms to the relevant ethical guidelines for human research.

Blood samples

Fasting venous blood samples were collected from each woman to obtain plasma (EDTA tubes) and serum. Serum and plasma were prepared by centrifugation (1000 ***g*** at 20 min). Plasma was immediately used for glucose determination. Serum was aliquoted and frozen at −80°C for further analysis of blood parameters.

Determination of blood parameters

Serum TAG, total cholesterol and free cholesterol concentrations were determined using enzymatic methods, according to the manufacturer's instructions furnished with the kit (Boehringer). HDL-cholesterol was also measured by a kit (Boo Scientific). LDL-cholesterol concentrations were calculated according to Friedewald *et al.*[32]. All biochemical parameters were analysed on a Synchron CX7 Clinical System (Beckman). Plasma fasting glucose was determined by the glucose oxidase method with a glucose analyser (Beckman Instruments). Plasma glycosylated Hb (HbA1c) concentrations were determined by isolab column chromatography[33]. Insulin serum concentrations were determined using an Insulin IRMA kit (Immunotech; Beckman Coulter, Inc.) with a detection limit of 0·5 μIU/ml (3·4725 pmol/l). The inter-assay CV was 3·3 and 4 % for the 13 and 54 IU/ml (90·285 and 375·03 pmol/l) concentrations, respectively.

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Serum concentrations of urea, creatinine and C-reactive protein were analysed by routine standard techniques using an automated Synchron CX7 Clinical System (Beckman). Serum concentrations of IL-6 and TNF-α were measured with ELISA kits (Immunotech).

Oleic acid sensitivity analysis

Taste emulsions containing food grade oleic acid (Sigma) were prepared according to Chalé-Rush *et al.*[34]. EDTA (0·01 %, w/v) was added to prevent fatty acid oxidation. The emulsions were sonicated for 4–5 min in a Labo-Modern sonicator at 4°C in an ice bath. Samples were stored in opaque polypropylene tubes and used for testing within 48 h of preparation. Control samples were prepared in the same way but without added oil.

The women were called on a stipulated date and advised to arrive early in the morning without having eaten breakfast (fasting state). The subjects were weighed, and a blood sample was drawn before the preference test to assess blood parameters. Taste preference tests for dietary lipids were performed by employing oleic acid at different ascending concentrations (0·018, 0·18, 0·37, 0·75, 1·5, 3, 6 and 12 mmol/l) by using a three-alternative forced choice (3-AFC) method[35]. According to the 3-AFC method, the patients were instructed to taste, one by one, three solutions; two of the solutions contained a control substance (acacia gum, 0·01 %), and the third one contained oleic acid in a solution that also included acacia gum (0·01 %). The acacia gum was used to mimic the textural properties of oil in the control solution.

We increased the concentration of oleic acid in the test solution when a single incorrect response was given, and we decreased the quantity of this fatty acid after two correct responses, in accordance with the method described by Pepino *et al.*[19]. A reversal in the response was considered when the concentration sequence changed direction. The procedure was terminated when there were four reversals that met the following two criteria. First, there could not be more than two dilution steps between two successive reversals. Second, the series of reversals could not form an ascending pattern. The threshold concentration was calculated as mean of log values for the last four reversals. To avoid visual and olfactory cues, the testing session was conducted under red light and participants used nose clips. The women were not allowed to drink the solutions; rather, they had to spit out each solution after keeping it in their mouths for a few seconds. If they responded that they observed no difference in the taste sensation, we increased the concentration of oleic acid. If they were able to detect a difference, it meant they were capable of detecting the presence of 'fatty taste'.

Genotyping analyses

Genomic DNA was extracted from 5 ml of whole blood with the use of a commercially available DNA isolation kit (Wizard Genomic DNA purification kit; Promega Corporation) according to the manufacturer's protocol. Genotyping of *TNF-α* −308 G/A (rs1800629), *IL-6* −174 G/C (rs1800795) and *CD36* A/G (rs1761667) was performed according to methods that have been previously used by our laboratory[36,37]. The PCR primers for the three genotypes were as follows: (*TNF-α*: 5′-AGG CAA TAG GTT TTG AGG GGC AT-3′ and 5′-CGG GGA AAG AAT CAT TCA ACC AG-3′; *CD36*: 5′-CAA AAT CAC AAT CTA TTC AAG ACCA-3′ and 5′-TTT TGG GAG AAA TTC TGA AGA G-3′; *IL-6*: 5′-ACT TTT CCC CCT AGT TGT GTC TTT C-3′ and 5′-AGA ATG AGC CTC AGA CAT CTC CAG T-3′). PCR amplification reactions were performed in a Veriti thermal cycler (Life Technologies). After initial denaturation for 3 min at 95°C, DNA was subjected to further amplification as follows for *TNF-α* and *IL-6*: denaturation for 30 s at 95°C, annealing for 30 s at 66°C and extension for 30 s at 72°C. After thirty-five cycles, a final extension for 5 min at 72°C was used. Amplified DNA was digested by either endonuclease Ncol (*TNF-α*) or TaqI (*IL-6*) and further incubated at either 37°C for 16 h (*TNF-α*) or 65°C for 5 h (*IL-6*). For *CD36*, the conditions were as follows: denaturation for 30 s at 95°C, annealing for 30 s at 56°C and extension 30 s at 72°C. After forty cycles, a final extension for 5 min at 72°C was used. Amplified DNA was digested by Hha1 at 37°C for 30 min. The digestion products were analysed by 2 % (w/v) agarose gel electrophoresis (Elisabeth Pharmacon) containing ethidium bromide, and DNA fragments were visualised under UV light. The following fragments were detected for *TNF-α*: 264 bp (GG genotype), 264 and 284 bp (AG genotype) and 284 bp (AA genotype). For *IL-6*, two fragments of 24 and 180 bp (G allele) and an unrestricted fragment of 204 bp (C allele) were obtained. Two fragments of *CD36* (138 and 52 bp) in the presence of the G allele were visualised, and an unrestricted fragment (A allele) had a length of 190 bp.

Statistical analysis

CSS Statistica software (StatSoft) was used for statistical analysis. An ANOVA was used for correlation of the different parameters and genotypes. A Kruskal–Wallis test was used for one-way analyses on ranks. The χ^2 test was used for the comparison of genotype frequencies. Fisher's exact test was used for the comparison of allelic frequencies. For correlation studies, we used Pearson's correlation coefficient method. Dunn's method was used for all pairwise multiple comparisons between the AA, AG and GG genotypes and the detection thresholds.

Results

Subject characteristics

Table 1 shows the anthropometric measures and concentrations of different blood parameters in the present cohort of obese Tunisian women (*n* 203). The values of glucose, insulin, urea, creatinine, cholesterol, HDL, LDL, HbA1c, apoA1, apoB, insulin and C-reactive protein were within normal ranges for obese women. Serum TAG, IL-6 and TNF-α concentrations were higher in the women as compared to previously reported control values[38,39].

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Table 2. Genotype frequencies in obese Tunisian women

Gene	SNP ID	Genotype	All subjects	Frequencies
CD36	rs1761667	GG/AG/AA	42/102/59	0·21/0·50/0·29
TNF-α	rs1800629	GG/GA/AA	140/56/7	0·69/0·27/0·04
IL-6	rs1800795	GG/GC/CC	146/47/10	0·72/0·23/0·05

CD36, cluster of differentiation 36.

CD36 *genotype and oleic acid detection thresholds*

Table 2 shows the genotype frequencies of three polymorphisms in the present cohort of obese women. Fig. 1 shows that the subjects with the GG genotype of the *CD36* gene had thresholds for oleic acid detection that were 3·3 times lower than those of subjects with the AA genotype (95 % CI of relative risk 2·5032, 4·4298, OR 9·9615; 95 % CI of OR 6·2101, 15·9793). We did not observe a statistically significant difference in the taste detection thresholds of subjects with the AG or the AA (or GG) genotypes. It is also noteworthy that some subjects, which have been termed non-tasters, could not detect fatty acid even at the highest concentration. There were a total of four non-tasters in the AA, AG and GG genotypes of *CD36* gene (Fig. 1).

Association between cholesterol, LDL and glycosylated Hb and CD36 *polymorphism*

Fig. 2(a) and (b) shows that cholesterol and LDL concentrations were significantly lower in subjects with the *CD36* GG and AG genotypes than in subjects with the AA genotype ($P<0·01$). Interestingly, the women with the GG genotype exhibited higher HbA1c plasmatic concentrations than did those with the AA genotype ($P<0·05$) (Fig. 2(c)).

CD36 *genotypes and TNF-α and IL-6 concentrations*

Serum concentrations of TNF-α was higher in women with the *CD36* AA genotype as compared to subjects with the GG genotype (Fig. 3(a)). Interestingly, serum IL-6 concentrations were lower in women with the AA and AG genotypes than they were in women with the GG genotype (Fig. 3(b)).

Association between TNF-α *polymorphism and creatinine serum level and association between* IL-6 *polymorphism and IL-6 serum level*

Fig. 4 shows that the women with the *IL-6* GG and *TNF-α* GG genotypes exhibited higher serum IL-6 and creatinine concentrations, respectively, than did those with the *IL-6* CC and *TNF-α* AA genotypes. Moreover, we did not observe a statistical association between the *TNF-α* SNP and serum TNF-α concentrations ($P>0·05$).

Discussion

Evidence suggests that there might be a sixth taste modality that is devoted to the oro-gustatory perception of dietary lipids[14,40]. Hence, it seems imperative to explore and better understand the mechanisms that underlie the oro-gustatory detection of dietary fat in order to help prevent and treat obesity[5,41]. A number of studies have suggested that lingual CD36, a glycoprotein that is highly expressed in circumvallate papillae, is implicated in the perception of dietary fat taste[6,12–15]. In the present study, we confirm that obese women with the *CD36* AA genotype (rs1761667) possess higher thresholds for lipid taste sensitivity than do those with GG genotypes.

Keller *et al.*[20] have provided preliminary evidence that CD36 is involved in human oral fat perception and the human attraction to added fats and oils in food. Pepino *et al.*[19] have demonstrated that *CD36* gene polymorphism, which results in a decrease in the gene's expression, is responsible for an increase in the oral detection threshold of dietary lipids in obese subjects. Aside from the present study, no confirming or refuting report is available on this subject, particularly from developing countries where diets are rich in fat. The present data strongly suggested that the oro-sensorial perception of fat taste is altered in some obese subjects. Indeed, we showed that the A allele of *CD36* rs1761667 polymorphism in obese women, which was previously associated with decreased expression of the CD36 protein, is associated with a high oro-gustatory threshold detection for oleic acid. Conversely, the subjects with the G allele were more sensitive in their oleic acid lingual detection as compared to the subjects with the A allele. These data corroborate not only the clinical findings of Pepino *et al.*[19] but also experimental data where an association between *CD36* gene expression and oral fat detection has been demonstrated[42]. Mice with partial *CD36* gene knockout ($CD36^{+}/^{-}$) had lower CD36 protein expression and a lower oral fat detection threshold than wild type animals ($CD36^{+}/^{+}$). The CD36 knockout ($CD36^{-}/^{-}$) failed to exhibit a spontaneous preference for fat.

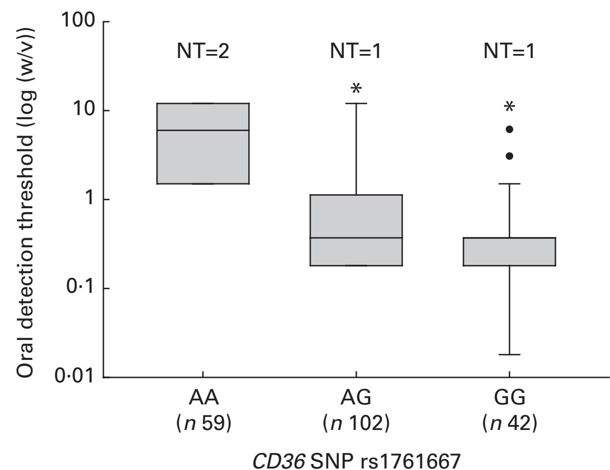

Fig. 1. Oleic acid detection thresholds in obese Tunisian women. The women (*n* 203) had either the AA genotype (*n* 59) or the GG (*n* 42) or AG (*n* 102) genotype of the cluster of differentiation 36 (*CD36*) gene. The figure shows the box plots of the medians, first and third quartiles, standard deviations and extreme values. The difference between the three groups was statistically significant ($P<0·001$; Kruskal–Wallis test). * Median value was significantly different from that of the AA genotype ($P<0·05$; one-way ANOVA). The difference between the AG and GG genotypes was not statistically significant. NT, non-tasters.

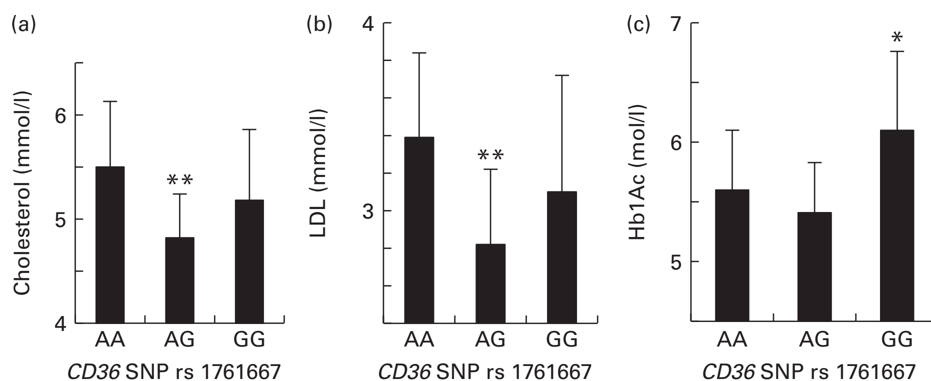

Fig. 2. Association between blood parameters and cluster of differentiation 36 (*CD36*) SNP in obese Tunisian women. Concentrations of cholesterol (a), LDL (b) and glycosylated Hb (HbA1c) (c) in women with the AA, AG or GG genotype of the *CD36* gene. Values are means, with standard deviations represented by vertical bars. Mean value was significantly different from that of the AA genotype: * $P<0.05$, ** $P<0.01$ (one-way ANOVA).

A low detection threshold for fat in the present study may not have been caused by the low expression of α-gustducin, a marker of taste receptor cells. Indeed, alteration in *CD36* expression is not related to changes in α-gustducin expression[42]. Moreover, α-gustducin knockout mice, like wild type animals, exhibited an unaltered preference for dietary fat[43]. It is possible that other proteins which are likewise involved in fat taste detection, such as GPR120, might also participate in low-fat taste sensitivity[44]. However, the CD36 and GPR120 receptors seem to be differently regulated in lipid taste perception[18,44].

In the present study, we also observed that some of the subjects failed to detect oleic acid in the emulsions. These subjects were defined as non-tasters, and they were also reported by Kamphuis *et al.*[9]. The mutation responsible for gustatory insensitivity to fatty acid in non-taster subjects deserves further in-depth study.

We performed the present study on Tunisian obese women (who probably eat an above-average amount of fatty food due to cultural customs in Tunisia), because it has been shown that some obese subjects had a low sensitivity to oleic acid[35,45]. Oral and gastrointestinal sensitivity to oleic acid are related to each other, and they are decreased in obese subjects[35]. Nonetheless, the present association studies cannot distinguish whether the decreased sensitivity to fat in obese women is a cause or a consequence of obesity. However, Stewart *et al.*[11] have shown that oral sensitivity towards oleic acid in lean subjects is decreased with a high-fat diet and increased with a low-fat diet. Brennan *et al.*[46] have reported that acute dietary restriction in obese subjects enhances their gastro-intestinal sensitivity to fat, and this is associated with an increased effect of fat on satiation. In addition, a high-fat diet has been shown to decrease the expression of *CD36* in mice[42].

We observed an association between the *CD36* AA genotype and high serum levels of cholesterol and LDL in obese women. Because the *CD36* A allele was previously associated with reduced expression of the *CD36* gene, it is possible that high blood lipid concentrations are the result of their curtailed uptake by adipocytes that also express CD36; in this case, it acts as a fatty acid transporter[47,48]. Moreover, *CD36* gene polymorphisms have been significantly associated with high TAG concentrations among ethnic Chinese in Taiwan[49].

Interestingly, obese women with the CD36 G allele had higher plasma HbA1c concentrations than women with the A allele. These observations corroborate the findings of Rać *et al.*[50], who have shown that the GG genotype was significantly associated with higher HbA1c concentrations as compared to the AA genotype of *CD36* in obese children.

We observed that obese women had high IL-6 and TNF-α serum concentrations. Homozygous women with the *CD36* AA genotype had higher TNF-α serum concentrations than did those with the GG or AG genotypes. Conversely, IL-6 serum concentrations were higher in women with the GG genotype than they were in women with the AA or AG genotypes. The importance of the association of high serum levels of TNF-α with the *CD36* AA genotype and the association of high serum levels of IL-6 with the *CD36* GG genotype is not well understood. These cytokines play a key role in the regulation of insulin sensitivity in subjects who are suffering

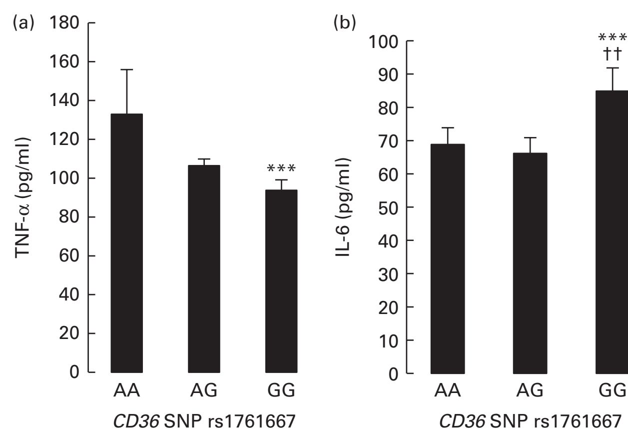

Fig. 3. Serum TNF-α (a) and IL-6 (b) concentrations in obese Tunisian women with the AA, AG or GG genotype of the cluster of differentiation 36 (*CD36*) gene. The serum concentrations of cytokines were determined as described in the Materials and Methods section of the present paper. Values are means, with standard deviations represented by vertical bars. *** Mean value was significantly different from that of the AA genotype ($P<0.001$; one-way ANOVA). †† Mean value was significantly different from that of the AG genotype ($P<0.01$; Fisher's exact test).

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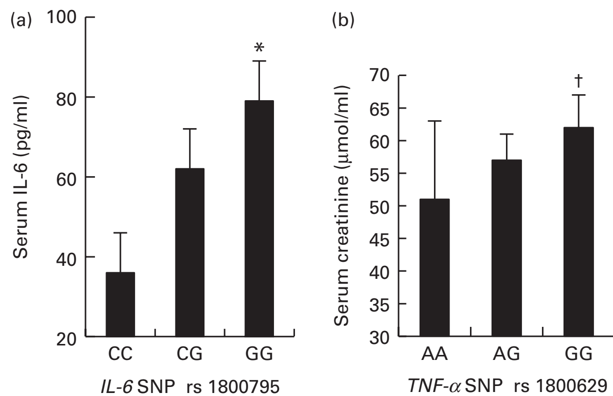

Fig. 4. Serum IL-6 (a) concentrations in obese Tunisian women with the IL-6 polymorphisms CC, CG or GG. Serum creatinine (b) concentrations in obese Tunisian women with the TNF-α polymorphisms AA, AG or GG. Values are means, with standard deviations represented by vertical bars. * Mean value was significantly different from that of the CC genotype ($P<0·05$; Fisher's exact test). † Mean value was significantly different from that of the AA genotype ($P<0·05$; one-way ANOVA).

from obesity and metabolic syndrome[51]. The SNP of these cytokines have been suggested to predispose for obesity[52]. The homozygous subjects with the GG genotype exhibited high serum IL-6 concentrations. These observations are in close agreement with the results of Pereira *et al.*[53], who studied the association between the *IL-6* gene and plasma IL-6 concentrations in community-dwelling and institutionalised older women. Those authors reported that women with the *IL-6* GG genotype had high IL-6 serum concentrations. High serum IL-6 concentration in homozygous subjects with the GG genotype might take part in increased fat oxidation in response to fat load in obesity, as has been suggested elsewhere[54]. With regards to TNF-α, we noticed a positive relationship between circulating creatinine concentrations and the *TNF-α* GG genotype, which indicates that the present subjects might be at risk for renal complications. Chang *et al.*[55] have shown that the G allele of the *TNF-α* gene was associated with high serum creatinine concentrations that increased the risk for contrast-induced nephropathy. We also observed a significant association between *IL-6* and *TNF-α* gene polymorphisms in obese women, which indicates that inflammatory status, as indicated by pro-inflammatory cytokines, is a key element of obesity in these women. Curtis & Singh[56] have likewise shown that the SNP of these two cytokines predispose for obesity.

Finally, we can state that a major value of the present study is that it validates the importance of a common *CD36* SNP rs1761667 in obese women. The present results must be confirmed by additional studies in other developing countries. It is also possible that in the present study, there might be an influence of female sex hormones on fat taste perception and other parameters. At this stage, it is difficult to determine whether oral fat perception sensitivity affects fat intake or body weight. Future studies are needed to answer these important questions. The stimulation of taste receptors, such as CD36, by synthetic fatty acid analogues within the oral cavity may provide a new target for obesity treatment.

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The authors' contributions are as follows: N. A. K. designed the research (project conception, development of the overall research plan and study oversight); I. M. conducted the research (hands-on conduct of the experiment and data collection); N. A. K. and O. S. wrote the manuscript; O. S. supervised the SNP research and statistical analysis; J. P. completed the technical part of the SNP analysis and participated in writing the manuscript; A. A., M. F., A. B. and M. Z. provided the facilities in the sample collections; N. A. K. and Z. T. supervised the study. All authors have read and approved the final content of the manuscript.

The authors declare no conflicts of interest.

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