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Association of adiponectin gene polymorphism and cytokines levels in obese patients with COVID-19

Huda Younis Abood
Department of medical chemistry, college of medicine, university of AL-Qadisiyah
*Corresponding author email: hudaalanbuky@gmail.com

Ferdous A. Jabir
Department of medical chemistry, college of medicine, university of AL-Qadisiyah

Mohammed Abdulwahab Ati Al-Askeri
College of biotechnology, university of AL-Qadisiyah

Abstract---Covid-19 is a genus of enveloped, positive-sense, single-stranded RNA viruses with a high degree of genetic diversity. They induce a variety of disorders affecting the respiratory, gastrointestinal, hepatic, and nervous systems in humans and animals, with different manifestation and severity. Several risk variables, including older age, male gender, type 2 diabetes mellitus, hypertension, coronary artery disease, and obesity (a higher body mass index (BMI)), are known to predict a worse course of covd-19 infection. Obesity is the result of the accumulation of an excessive amount of fat in the body and the condition arises from an imbalance between the amount of energy stored by increased food intake and the amount of energy expended as physical activity. The aims of study evaluate the effect of covid-19 on cytokines levels in obese patients. As well as, study the association of adiponectin gene polymorphism to the risk of infection with covid-19 in obese patients. This study is cross sectional. Which include conducted in isolation wards at Al-Amal Hospital and Al-Hakim hospital in Al-Najaf City for the period extending from November 2021 to the end of March 2022, 60 sample were collected from diagnosed patients with covid-19 and obese with BMI more than 30 (G1) . On the other hand, 60 sample of individuals that suffering from obesity where BMI more than 30 and recovered from covid-19 for more than six months (G2). The variables were measured in this study: IL-6, IL-10, TNF-a and Leptin, and also gene polymorphism of adiponectin (rs266729) gene. The results showed that there were significant differences for (IL-6, IL-10 and TNF-a), in obese patients with covid-19 compared to obese recovery patients except leptin show no significant difference. The gene polymorphism of adiponectin show no

significant difference between obese patients with covid-19 and obese recovery from covid-19. In conclusions obese people are the most risk factor to covid-19 infection due to association of obesity with change in BMI and increase Adipose tissue mass in obese individuals, this change represent a leading cause to inflammatory alteration, as shown increase inflammatory markers(IL-6, IL-10 and TNF-a) in recovery obese individuals from covid-19. So that the inflammatory biomarkers are good predictors for accelerate the infection with covid-19 in obese people. While gene polymorphism study of SNPs of adiponectin (rs266729) gene represent a weak maker for covid-19 disease.

Keywords---COVID-19, obesity, adiponectin, cytokines.

Introduction

Coronavirus disease 2019 (COVID-19), an emerging acute respiratory disease, is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This disease starts in Wuhan city in china, in the late December 2019. A study of the first cases in China indicates the average incubation period between 2 to 7 days and the longest incubation period of 12.5 days. The World Health Organization named the disease coronavirus disease 2019 (COVID-19) and subsequently declared it a pandemic due to the widespread infectivity and high contagion rate. Human coronaviruses typically cause respiratory and enteric infections. The new coronavirus has become a worldwide health threat, with sporadic cases reported globally From December 2019 till now, the disease, which resulted in several millions cases with many deaths. The common symptoms include fever, dry cough, and tiredness though some individuals remain asymptomatic throughout the period. Asymptomatic patients are considered to be a potential source of infection. the key instruments for confirmed diagnosis of the infection are virus nucleic acid real-time polymerase chain reaction (RT-PCR), CT imaging, and certain hematology parameters.

several risk factors prognosticate an unfavorable disease course of COVID-19 are known, including higher age, male sex, type 2 diabetes mellitus, hypertension, coronary artery disease, and obesity (a higher body mass index (BMI). The worldwide prevalence of overweight and obesity has doubled since 1980 to an extent that nearly a third of the world population is now classified as overweight or obese. Obesity adversely affects nearly all physiological functions of the body and comprises a significant public health threat. It increases the risk for developing multiple disease conditions, such as diabetes mellitus, cardiovascular disease, several types of cancers, an array of musculoskeletal disorders, and poor mental health. and increase risk factor for covid-19. The World Health Organization (WHO) defines overweight and obesity as abnormal or excessive fat accumulation that presents a risk to health. The body mass index (BMI) is body assessment for obesity, calculated by dividing the body weight in kilograms by the square of height in meters, is a simple metric used to indicate overall body fatness;

- normal BMI range as 18.5 to 24.9
- overweight range as BMI ≥ 25 kg/m2
- obese range as BMI ≥ 30 kg/m2
- severe obesity range as BMI ≥ 40 kg/m2

Measurements and comparisons of waist and hip circumference can also provide some information regarding risk factors associated with weight. The higher of the ratio, the greater chance for weight-associated complications. White adipose tissue (WAT), once regarded as the major site of energy storage and homeostasis, is now known to be an endocrine organ producing numerous biologically active molecules and hormones, one of the most important being adiponectin. Mainly secreted by adipocytes, adiponectin is also produced to some extent by bone marrow, osteoblasts, fetal tissue, myocytes, cardiomyocytes, and salivary gland epithelial cells. In contrast to other known adipokines, adiponectin is inversely related to body mass index (BMI) and central adiposity; the strongest negative correlation has been observed with waist-to-hip ratio. Undoubtedly, a feedback loop regulates it at transcriptional, translational or post-translational level. A similar trend in downregulation of its receptors AdipoR1 and AdipoR2 have also been observed. Normal levels of adiponectin as well as its receptors are re-established post weight/fat loss. Though a number of mechanisms have been proposed, none of them precisely explain the feedback mechanism in adiponectin regulation. An obese state is a situation of chronic inflammation in the body characterized by a marked increase in the levels of inflammatory cytokines IL6, IL8, TNFα, and leptin, which are directly known to inhibit adiponectin transcription.

Leptin, located on chromosome 7, is a 167-amino acid polypeptide, and molecular weight is 16 kD. It is mainly synthesized and secreted by WAT. However, the placenta, ovary, skeletal muscle, mammary epithelium, bone marrow and lymphoid tissue could also express leptin. Much of the research on leptin performed during the early days focused on its role in regulating energy homeostasis and obesity at the level of the central nervous system. The role of leptin in the regulation of energy homeostasis was demonstrated by observing leptin-deficient patients, who develop hyperphagia and obesity during childhood, and can be aided by leptin replacement therapies that suppress appetite and increase energy expenditure. This attracted a lot of interest toward the clinical use of leptin for the treatment of obesity in humans. However, most obese subjects are not deficient in the leptin gene, and the circulating levels of leptin are elevated compared to those in non-obese subjects. Paradoxically, these obese subjects remain obese, reflecting a state of leptin resistance that leads to the intake of extra calories and prevents sustained weight loss. The mechanisms involved in leptin resistance have not been clarified since the discovery of leptin in 1994. Different mechanisms have been suggested, such as elevated levels of C-reactive protein, the downregulation of the leptin-activated signal transduction pathway, or a decrease in histone deacetylase activity. However, alterations in the transport of leptin to the brain through the blood–brain barrier (BBB), a mechanism that has not been completely deciphered, seem to play a fundamental role.

Cytokines are proteins with low molecular weight discharged by activated immune cells and stromal cells and having vital activity via linking to cognate receptors on

the cellular membrane. Cytokines can be formed by many cells, constantly WBCs, which modulate many normal and pathological processes such as acquired immunity, innate immunity, and a plethora of inflammation. Cytokines prevent the formation exaggeration and differentiation of many related target cells depending on antigen inducing which may lead to moderation in the activities of many other cells involved in the immunological process especially mast cells, macrophages, natural killer cells B cells, and T cells. Thus, cytokines are considered released polypeptides with differentiation, and growth, that modulates and specify the direction of immunological processes.

There are currently six families of cytokines classified by structure as well as function, including interleukins (with several subfamilies based on peptide and receptor homology), interferons (IFNs), tumor necrosis factor family (TNF), chemokines, growth factors, and colony-stimulating factors (CSFs). Other categorization strategies have been utilized, and cytokines can be classified based on the structural homology of their receptors. Almost all of the above families of cytokines can be grouped into class I or class II (125). Viral infection activate immune response in the host leading to a “cytokine storm”. The novel coronavirus infection elicits a similar response in the host. This often involves the interplay of various chemokines, colony-stimulating factors, interferons (IFNs), interleukins (ILs) as well as tumor necrosis factor-α (TNF-α). The cytokine storm is correlated to the severity of the infection and often causes extensive damage or injury. Furthermore, it is also considered as a leading cause of ARDS, and multi-organ failure, which are closely associated with the severity and progression of covid-19. Moreover, the cytokine storm and associated complications are the major cause of death in covid-19 patients. Many studies suggest the clinical characteristics of the cytokine storm in covid-19 patients. In the extremely severe patients, elevated levels of IL-2 receptor (IL-2R), IL-6 as well as IL-10 were observed. Moreover, a gradual reduction in the absolute count of CD4+ T, CD8+ T, and B cells was also observed as the severity of the disease progressed. These findings suggested that there is a correlation between immune response and severity of covid-19 progression.

Materials and Methods

This study is cross sectional study and included 120 individuals included:

- G1, 60 individuals that suffering from obesity (BMI more than 30) and recovered from covid-19 for more than six months.
- G2, 60 patients diagnosed with Covid-19 who were suffering from obesity (BMI more than 30), All patients were admitted to the quarantine section of Al-Amal Hospital and Al-Hakim hospital in Al-Najaf City for the period extending from November 2021 to the end of March 2022.

Ethical Issues

For this study, all samples were obtained from participants. All of the patients expressed their willingness for their specimens to be used in this study. All ethical concerns were addressed, and the research was carried out with the permission of

the hospitals. The names and characters, personal information, and even the illnesses and medical information of the patients were kept hidden.

Included criteria

Any person whose BMI more than 30 and infected with covid-19 has been confirmed by a PCR test or CT scan examination, or by a specialist doctors diagnosis of the disease.

Collection of Samples

Whole venous blood samples under aseptically collected by venous puncture using sterile 5ml disposable syringe. Blood divided to two parts;

- Part one, 1.5 ml whole blood insert to EDTA plastic tube and stored in deep freezer in - 20 ℃ until they used in T-ARMS-PCR assay for adiponectin gene polymorphism analysis.
- Part two, 3.5 ml blood was evacuated in a gel and clot activator vacuum tubes that were centrifuged at 3000 rpm for 5 minutes. The serum was separated in a new plain tube for biochemical test by ELISA for (IL-6, IL-10,TNF-a & leptin).

Biochemical methodology (ELISA)

The serum samples of patients were obtained for screening of the concentration of IL-6, IL-10,TNF-a & leptin by ELISA using commercial kits (Bioassay technology laboratory, china).

Tetra Primer Amplification Refractory Mutation System PCR (tetra ARMS-PCR)

In this study, we designed a Tetra amplification refractory mutation system polymerase chain reaction (T-ARMS-PCR) for detection of polymorphisms of adiponectin. This method is simple, rapid and sensitive for the detection of single nucleotide polymorphism. The adiponectin genomic sequence was obtained from. PCR amplification for genotyping T-ARMs PCR was carried out, briefly, four primers of each SNP were designed, Forward Outer, Forward Inner, Reverse Outer, and Reverse Inner. (Forward outer: 5`- GGA CTG TGG AGA TGA TAT CTG GGG GGC A-3`, Reverse outer: 5`- TGG CCT AGA AGC AGC CTG GAG AAC TGG A-3`), and the two allele specific internal primers were (Forward inner (C allele): 5`- CTT GCA AGA ACC GGC TCA GAT CCT CCC-3`, Reverse inner (G allele): 5`- GAG CTG TTC TAC TGC TAT TAG CTC TGC-3`). PCR mixture of 25 µl was made consisting of 12.5 µl master mix, 1.25 µl of each forward and reverse primer, 7.5 µl of template DNA, and without of ddH2O. The PCR amplification conditions were; initial denaturation 95ºC for 5 min, proceeding with 38 cycle of denaturation at 95ºC for 30 s, annealing at 62ºC for 30 s, extension 72ºC for 30 s, and final extension at 72ºC for 5 min. The amplified PCR products was run on 1.5% of agarose gel.

Statistical analysis

The data analysis for this work was generated using The Statistical Package for the Social Sciences software, version 28.0 (IBM, SPSS, Chicago, Illinois, USA) and the Real Statistics Resource Pack software for Mac (Release 7.2) of the resource pack for Excel 2016. Results of all tests with p-values <0.05 (two-side) were considered to be statistically significant.

Results

This study was examined four biochemical markers (IL-6, IL-10, TNF-a and Leptin). All markers were shown a significant difference except leptin as show in table (2). The IL-6 level in G1 was higher than its level in G2, Mean ± 2SD of IL-6 level in G1 (56.752± 8.906 ng/L) versus (52.954 ± 10.388 ng/L) and the difference was highly significant (p = 0.034). The IL-10 level in G1 was higher than its level in G2, Mean ± 2SD of IL-10 level in G1 (110.733 ± 23.006 pg/ml) versus (96.966 ± 32.434 pg/ml) and the difference was highly significant (p = 0.008). The TNF-a level in G1 was higher than its level in G2, Mean ± 2SD of TNF-a level in G1 (66.923 ± 11.706 ng/L) versus (54.491 ± 13.563 ng/L) and the difference was highly significant (p<0.001). The leptin level in G1 was higher than its level in G2, Mean ± 2SD of leptin level in G1 (1.143 ±0.296 ng/ml) versus (1.079 ±0.314 ng/ml) and the difference was nonsignificant (p = 0.252).

Table 2
The association between serum biochemical markers in G1 and G2

Variables	G1	G2	P-value
IL-6	56.752± 8.906	52.954 ± 10.388	0.034 [S]
IL-10	110.733 ± 23.006	96.966 ± 32.434	0.008 [S]
TNF-a	66.923 ± 11.706	54.491 ± 13.563	<0.001 [S]
Leptin	1.143 ±0.296	1.079 ±0.314	0.252 [NS]
Results are presented as mean ± SD, p<0.05 considered significantly different, [S]= Significant, [NS]= Non significant, independent T-test			

In table (3), A logistic regression was performed to ascertain the effects biomarkers on the likelihood that participants have Covid-19 infection. The odd ratio of markers in both groups represent the risk factor of each independent variables and their clinical importance in covid-19 infection. Among covid-19 patients groups IL-6 were shown a high risk factor in covid-19 infection. The logistic regression model was statistically significant, OR (1.059) and p < 0.067, while leptin not showing any statistical significant among covid-19 groups.

Table 3
The risk factor of some biochemicals markers by using binary logistic regression

Biomarkers	Odds Ratio	95% Confidence Interval Range (Lower-Upper)	P Value
IL-6	1.059	(0.996 – 1.126)	0.067
IL-10	0.977	(0.954 – 1)	0.051

TNF-a	0.903	(0.860 – 0.948)	<0.001
Leptin	3.583	(0.403 – 31.822)	0.252

Receiver operating characteristics (ROC) curve of diagnostic markers in G1

In each patients group, ROC curves were performed for levels IL-6, IL-10, TNF-a & Leptin. The AUC and cut-off values were calculated according to their specificity and sensitivity as predictive factors. IL-6 had the high AUC, which was 0.6 [95% Cl=0.513 - 0.715; Sensitivity% =82%; Specificity% =40%; Cut-off points =49.98]. IL-10 had the high AUC, which was 0.8 [95% Cl=0.677 - 0.857; Sensitivity% =90%; Specificity% =55%; Cut-off points =95.326]. TNF-a had the high AUC, which was 0.8 [95% Cl=0.703 - 0.871; Sensitivity% =90%; Specificity% =48%; Cut-off points =53.66]. Leptin had the high AUC, which was 0.6 [95% Cl=0.490 - 0.695; Sensitivity% =70%; Specificity% =43%; Cut-off points =1.023].as shown in table (4).

Table 4
AUC, optimal threshold, Sensitivity and specificity of (IL-6, IL-10, TNF-a and Leptin) levels obtained by the ROC curves for G1

Test variables	AUC	Sensitivity %	Specificity %	Cut-off points	Accuracy %	CI (95%)	P value
IL-6	0.6	82%	40%	49.98	60.8%	0.513 - 0.715	0.031
IL-10	0.7	90%	55%	95.326	65%	0.677 - 0.857	<0.0001
TNF-a	0.8	90%	48%	53.66	69.16%	0.703 - 0.871	<0.0001
Leptin	0.6	70%	43%	1.023	56.66%	0.490 - 0.695	0.080

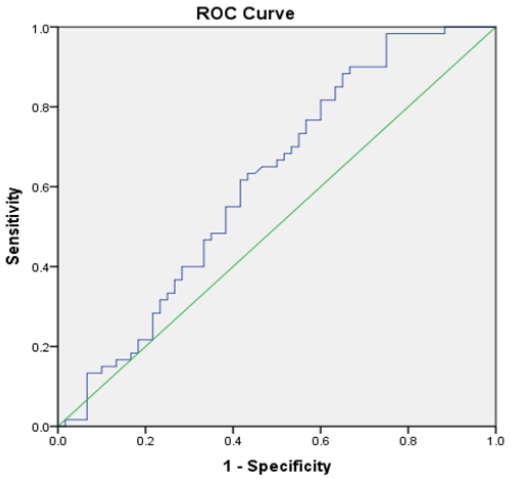

Figure 1. Receiver operating characteristics (ROC) curve analysis of IL-6 level in G1

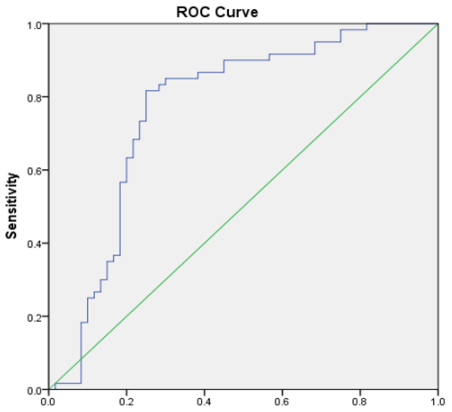

Figure 2. (ROC) curve analysis of IL-10 level in G1

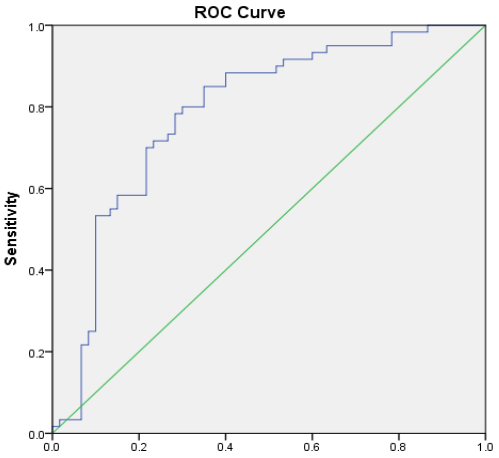

Figure 3. (ROC) curve analysis of TNF-a level in G1

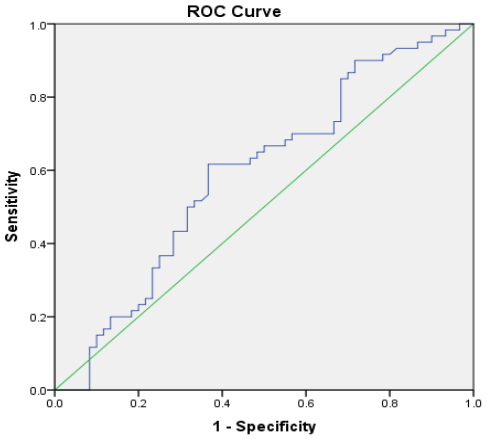

Figure 4. (ROC) curve analysis of leptin level in G1

Results of adiponectin (rs266729) gene polymorphism by allele specific PCR technique

The results of the PCR technique showed the success of all DNA amplification processes extracted from the G1 and G2 of the adiponectin gene after Perform electrophoresis on the agarose gel. It showed the diagnostic gene for adiponectin at molecular weight 299 bp, 155 bp and 201bp as revealed in figure (5).

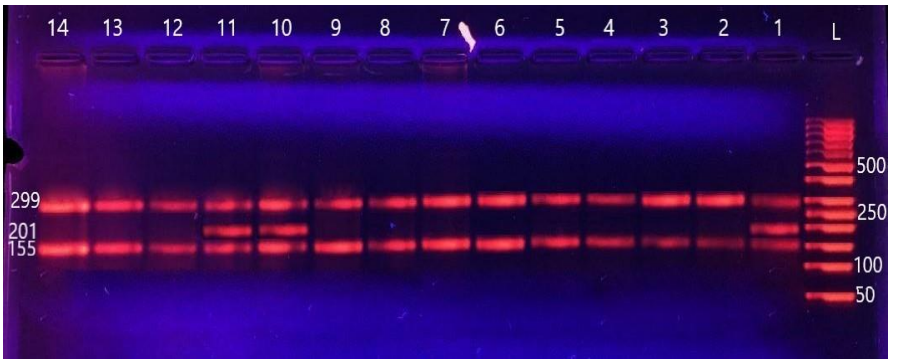

Figure 5. agarose gel electrophoresis for PCR product of adiponectin gene. product sizes were 299 bp for control band, 201 bp for G allele, and 155 bp for C allele. Lane number (1, 2, 3, 4, 5, 6, 7) for group1 and lane number (8, 9, 10, 11, 12, 13, 14) for group 2. Agarose 1.5% concentration, 75 V, stained with ethidium bromide. L, DNA ladder (50bp)(promega company)

Table (5) illustrates the examination 60 individuals of G1 and 60 individuals of G2. The result of PCR analysis for amplification of adiponectin gene polymorphism was 3(5.0%) for GC , 56(93.3%) for CC genotype and 1(1.7%) for GG genotype in G1and 4(6.7%) for GC , 54(90.0%) for CC genotype and 2(3.3%) for GG genotype in G 2. this result indicate there is no significance difference through this gene in two groups.

Table 5
Associated factors of Adiponectin Genotype (single-nucleotide polymorphisms rs266729) in Obese compered to obese infected with Covid-19 group

Genotype	G1 N (%)	G2 N (%)	Odd ratios	95% CI	P value
GC	3 (5.0)	4 (6.7)	1.383	0.296 – 6.469	0.681[NS]
CC	56 (93.3)	54 (90.0)	1[a]		-
GG	1 (1.7)	2 (3.3)	2.074	0.183 – 23.545	0.555[NS]

Discussion

The immune system as a whole is impacted by obesity, with changes occurring in the quantities of cytokines and proteins, as well as the number of immune cells and their activity. This imbalance causes a condition of chronic inflammation, which in turn contributes to the development or severity of a number of different diseases. In relation to covid-19, whose extreme situations develop with an

intensive and extreme systemic inflammation (cytokines storms), Obesity-associated immune dysfunction may be a factor in the poorer clinical outcome. This is due cytokine storms are characterized by intense and severe systemic inflammation. Adipocytokines, and especially leptin, have a significant impact on the quantity of immune cells as well as their activity. They do this by having direct impacts on the metabolism of individual cells. In this setting, there may be a rise in the number of cytotoxic and effector T-cells (Th1 & Th7) as well as M1 macrophages, Although Treg cells and M2 macrophages may also decline at the same time. However, Other chemicals that are impacted by dietary status also have an effect on the immune system and on obese patients, this immunity may be either increased (TNF-a, IL-6, IL1B, IL-10, IL-1RA, IL-8, Visfatn, Resistn, MIF, MCP-1, MIP1 alpha & beta) or decreased (IL-33, Adiponectin) depending on the molecule. In addition, an imbalance between the actions of lymphocytes called Treg & Lymphocytes called CD17+ also adds to the proiflammatory state that seen in obesity.

This conclusion gives support to the results of this research, which showed that G1 had higher levels of IL-6, TNF-a, IL-10, and Leptin compared to G2, so that the decrease in cytokines levels in G 2 may be come from use of immune suppression drugs with covid-19 patients like The usage of corticosteroids is indicated for covid-19 that used for modulate the immune response in a broad variety of diseases like dexamethasone and cortisone. and tocilizumab that is immune biological anti IL-6 receptor use for prevent cytokine release that also known as Actemra that use as a possible treatment for covid-19. Vitamin D supplementation has also been shown, through a variety of mechanisms, to reduce the risk of covid-19, lowering amounts of pro-inflammatory cytokines, which are responsible for the inflammation that causes damage to the lining of the lungs and ultimately results in pneumonia. This also leads to the usage of combined anti-inflammatory and antiviral therapies.

Adiponectin is involved in a number of key metabolic and cellular functions throughout the body. Insulin sensitization and anti-inflammatory actions are its two primary roles in the body. In this study Adiponectin gene (rs266729) has no significant with covid-19. On the other hand, there is some evidence to suggest that the circulation levels of adiponectin are lower in people who are obese, The re266729 minor G allele of the adiponectin gene was related with decreased adiponectin levels, which indicated that there was a causal association between adiponectin and metabolic characteristics. According to meta-analysis that done in 2014 and find rs266729 is linked with an increased risk of obesity in individuals of Asian ethnicity (2CC+CG vs 2GG+GCG: OR = 0.77, 95% CI = 0.65–0.92).

Conclusion

- Obese people are the most risk factor to covid-19 infection especially in old age individual's due to inflammatory, hormonal and metabolic alteration.
- Obesity is associated with change in body BMI and increase Adipose tissue mass in obese individuals, this change represent a leading cause to inflammatory alteration, as shown increase inflammatory markers(IL-6, IL-10 and TNF-a) in obese individuals without covid-19 infection. So that the

inflammatory biomarkers are good predictors for accelerate the infection with covid-19 in obese people.

- Adiponectin (rs266729) gene represent a weak maker for covid-19 disease.
- Leptin test is leading cause to obesity whereas control eat and hunger, but represent weak maker to covid-19 disease.

Recommendation

- Clinicians caring for obese individuals that infected with covid-19 should aware of the inflammatory alteration that accompany with hormonal change like leptin and adiponectin.
- Covid-19 infection is a leading cause to many other systemic diseases, so that must use suitable medicine thoroughly and individually assessed with special attention paid to the kind and route of administration.
- More extended studies with other SNPs of adiponectin gene should be carried out to give more accurate picture about gene polymorphism studies.
- Assessment of Gene expression of adiponectin gene that is an indicator of metabolic alteration in obese individuals.

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