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Association of the AGT rs699 and ACE rs1799752 Gene Polymorphisms with Obesity and COVID-19 Severity in a Cohort of Syrian Patients

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Abstract:

Background and Aim: The COVID-19 pandemic represents a sound example of inter-individual variations in disease severity ranging from asymptomatic and mild disease to severe illness, which may end up with organ failure and ultimately death. The genetic basis of the inter-individual disparities in disease severity is an area of rigorous research worldwide. This study aimed at investigating the proposed associations between disease severity and two gene polymorphisms, namely the rs699 in angiotensinogen (AGT) gene and the rs1799752 in the angiotensin converting enzyme 1 (ACE I) gene, due to the key regulatory roles of the encoded proteins in the Renin-Angiotensin-Aldosterone System (RAAS), and their linkage to risk factors (i.e., hypertension and obesity).

Study Subjects and Methods: We interviewed 54 subjects who had been previously diagnosed with COVID-19. Information on demographics, COVID-19 severity and comorbidities were collected. Patients were classified based on body mass index (BMI) into three categories; lean or normal weight (NW), overweight (OW), and obese (OB), and graded based on COVID-19 severity into mild, moderate and severe disease. Whole blood samples were drawn and genotyping was performed via electrophoresis (rs1799752) or standard sequencing (rs699) of the specific polymerase chain reactions (PCR) amplicons.

Results: Our findings revealed a higher frequency (41.2%) of the rs1799752 (I) allele in the OW and OB groups compared with the NW subjects (20%) (p= 0.024). The difference was more evident when limiting comparison to young (< 50 years) OW and OB versus NW individuals (50% versus 13.9%, respectively) (p= 0.0012). Females had higher frequency (70.8%) of the TC genotype and only (12.5%) of the CC, whereas males had disparate frequencies of (33.3%) and (40%), respectively (p= 0.018). The ID genotype was dominant (62.5%) whereas the DD genotype made only (18.75%) of the youth (< 50 years) in the OW and OB groups, compared with frequencies of (27.8%) and (72.2%), respectively, in the NW individuals (p= 0.012). Furthermore, the ID and DD genotypes constituted (50.0% and 41.2%), respectively, of the moderate and severe cases versus (35.0% and 50.0%), respectively, in the mild COVID-19 patients (p= 0.04). Eleven of the 34 (32.35%) moderate and severe cases of the TC-ID haplotype compared with only two cases of 20 (10.00%) in the group of mild disease. Conclusions: Our results suggest correlations between the I allele and ID genotype with obesity, and ID genotype and TC-ID haplotype with the COVID-19 severity in a cohort of Syrian patients.

KeyWords: Rs699 Polymorphism, Rs1799752 I/D Polymorphism, Hypertension, Obesity, COVID-19.

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ترافق التعددين الشكليين AGT rs699 و ACE rs1799752 مع البدانة وشدة داء كوفيد-19 لدى جمهرة من المرضى السوريين

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الملخص:

خلفيّة البحث وهدفه: تُقدّم جائحة كوفيد-19 مثالاً نموذجيّاً عن التباينات بين الأفراد في شدّة العدوى والتي تتراوح بين الأعراض الخفيفة وصولاً إلى الدّاء الشّديد، وانتهاءً بفشل الأعضاء والوفاة في نهاية المطاف. يشكّل الأساس الجيني للاختلافات في شدّة العدوى بين الأفراد محطّ اهتمام العديد من البحوث حول العالم. هدفت هذه الدراسة، إلى تحرّي الارتباطات المفترضة بين شدّة داء كوفيد-19 والتّعددين الشّكليين rs699 في جين الأنجيوتنسينوجين (AGT) وrs1799752 في جين الأنجيوتنسين 1(ACE) ، نظراً للأدوار التّنظيميّة الرئيسيّة للبروتينين المرمَّزين بهاتين الجينين لجملة الرينين أنجيوتنسين-ألدوستيرون (RAAS)، واحتمال ارتباطهما بعوامل خطورة أخرى (مثل ارتفاع ضغط الدم والبدانة). أفراد الدّراسة والطرائق: أُجريت مقابلات مع 54 فرداً من الجنسين شُخّصت لديهم عدوى سابقة بكوفيد-19 وجُمعت معلومات تتصل بالخصائص الديموغرافيّة وشدّة داء COVID-19 والأمراض المصاحبة. صُنّف أفراد الدراسة على أساس منسب كتلة الجسم (BMI) إلى مجموعات ثلاث: طبيعيّة الوزن(NW) ، وزائدة الوزن(OW) ، وبدينة(OB) ، كما صُنّفوا اعتماداً على شدة المرض إلى داء خفيف ومتوسطّ الشّدّة وشديد. بُزلت عيّنات الدم الكامل وأُجريَ التنميط الجيني عن طريق الرحلان الكهربائي (rs1799752) أو السّلسَلة المعياريّة (rs699) لمنتجات تضخيم الدنا النّوعيّة لتفاعل البلمرة المتسلسل (PCR)

النّتائج: بيّنت المقارنات التي عُقدت بين مجموعات الدّراسة تكراراً أعلى للأليل (I rs1799752) في أفراد مجموعتيOW و OB (41.2%) مقارنة بالأشخاص طبيعيي الوزن (20%)، وبفارقٍ معتدٍّ به إحصائيّاً(p= 0.024) ، وكان الفارق أكثر جلاءً لدى الاقتصار على الأفراد OW وOB بالمقارنة مع NW الشّباب (<50 عاماً) 50)% مقابل 13.9%، على التّرتيب .(p= 0.0012) (كان النّمط الجيني TC للأليل rs699 الأعلى تواتراً لدى الإناث (70.8%) مقابل تواتر منخفض للنمط الجيني CC (12.5%) مقارنة بتواترات متباينة لدى الذكور (33.3% و40%، على التّرتيب(، .(p= 0.018) هيمن النمط الجيني ID وبنسبةٍ قدرها 62.5%، في حين لم يتعدَّ تواتر النّمط الجيني DD نسبة 18.75% لدى الشّباب (< 50 عاماً) في مجموعتي OW وOB مقارنة بالتّواترات المسجّلة لدى الأفراد طبيعيي الوزن (27.8 و72.2%، على التّرتيب.(p= 0.012) بلغ تواتر النّمطين الجينيّين ID و41.2% DD50.0 و، على التّرتيب لدى الأفراد ذوي الحالات متوسطة الشّدّة والشديدة مقارنة بتواترات 35٪ و50٪، على التّرتيب، لدى أفراد الأعراض الطفيفة (p= 0.04) أخيرًا، كان 11 فرداً من أصل 34 (32.35%) في مجموعة الحالات المتوسطة والشديدة من النمط الفرداني TC-ID مقارنة مع فردين فقط من أصل 20 (10.00%) في المجموعة خفيفة الأعراض.

الاستنتاجات: تقترح نتائجنا وجود ارتباط بين كلٍّ من الأليل I والنمط الجيني ID مع البدانة، وارتباط بين كلٍّ من النمط الجيني ID والنمط الفرداني TC-ID مع شدة داء كوفيد-19 في مجموعة من مرضى كوفيد-19 السوريين.

الكلمات المفتاحيّة: التعدد الشكلي rs699، التعدد الشكلي I/D rs1799752، داء كوفيد-19، البدانة، ارتفاع التّوتّر الشّرياني.

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Introduction:

In recent years, the world has witnessed the outbreak of COVID-19 pandemic, which has a wide clinical spectrum, ranging from asymptomatic and mild disease to severe pneumonia and even acute respiratory distress syndrome ARDS [1]. Other complications included metabolic acidosis, severe heart damage, arrhythmia, septic shock, neurological manifestations, ocular, coagulopathy, secondary infections, multi-organ damage, and renal and hepatic dysfunction [2]. Furthermore, the viral infection was accompanied by a severe inflammatory response, which in many cases, led to severe lung damage that required admission to the intensive care unit and mechanical ventilation. It was also accompanied by an increased risk of multi-organ failure and death [3].

Several reports have addressed the role played by the renin-angiotensin-aldosterone system (RAAS) in the pathogenesis of COVID-19 disease [4,5], where angiotensin-converting enzyme ACE catalyzes the synthesis of angiotensin-II (Ang-II) from Ang-I, and then the ACE2 enzyme hydrolyzes Ang-II into Ang-1-7. Ang-II binds to the AT1 receptor, associated with vasoconstriction, fibrosis, inflammation, thrombosis, and other responses. On the other hand, Ang-1-7 binds to the AT2 receptor, resulting in dilation of blood vessels and reduction of fibrosis, inflammation, and thrombosis. In fact, ACE2 produces a protective response in the lung by reducing edema, permeability, and lung damage, while previous studies revealed that high ACE activity increases the risk of lung and cardiovascular diseases by increasing the activity of the Ang-II/AT1R axis [4]. Hence, ACE and ACE2 might play opposite roles in the homeostasis that leads to the risk of hypertension and cardiovascular disease. ACE is encoded by the ACE gene located on chromosome 17q23.3, while ACE2 is located on X chromosome [6].

While age, diabetes, and high blood pressure are well known risk factors for severe COVID-19, several reports in the past few years have revealed associations between COVID-19 severity and genetic variants in genes associated with RAAS, including ACE and ACE2 [7-10]. In fact, genetic variants in the ACE and ACE2 genes are associated with various diseases such as the risk of high blood pressure, heart disease, kidney failure, and lung disease. Rationally, any change in the expression and function of RAAS elements, for example, due to genetic differences, could lead to differences in the pathogenicity of COVID-19.

ACE rs1799752, also known as rs4646994 or rs4340, is a common insertion (I) – deletion (D) (indel) polymorphism with a 287-bp Alu-type sequence at intron 16 position, leading to elevated ACE activity and serum levels, and angiotensin-II levels in the carriers of the D allele [11]. It was suggested that such an elevated level of ACE could be a strong risk factor for cardiovascular and kidney diseases. A positive association between the D allele and ACE levels and acute respiratory distress syndrome has also been observed. However, its association with hypertension appears to depend on race [11].

Another important player in the RAAS system is angiotensinogen (AGT), which is a peptide hormone encoded by the *AGT* gene mapped on chromosome 1q42.2 [12]. Angiotensinogen is cleaved by renin and produces angiotensin-I, which is subsequently converted to angiotensin-II by ACE. A genetic variation in *AGT* gene, a single nucleotide polymorphism rs699 or M268T, is a T to C missense polymorphism on exon 2, replacing methionine residue with threonine at the 268 primary protein structure site, which is associated with increased plasma angiotensinogen levels and, hence, hypertension. Therefore, this variant is likely associated with increased susceptibility to COVID-19 [12].

Taken together, the involvement of either rs1799752 or rs699 in COVID-19 severity and mortality is still controversial, and it seems their effects are dependent on ethnicities and probably several other factors. In this report, we studied the association of the two polymorphisms, rs1799752 and rs699, with severity of COVID-19 disease in a cohort of Syrians with previously confirmed COVID-19 disease. We compared the frequency of the two polymorphisms based on categorization patients according to obesity, hypertension and COVID-19 severity.

Materials And Methods:

Subjects and Samples

This retrospective study included 54 non-related participants (24 females, 44.44%, and 30 males, 55.56%) with previously confirmed COVID-19 disease, either be PCR positive tests (22 cases, 40.74%) or positive chest X-ray in addition to clinical symptoms (22 cases, 40.74%) or positive Corona virus IgG (10 cases, 18.52%).

The severity of SARS-CoV-2 infection was classified according to the latest "COVID-19 Treatment Guidelines" issued by the American National Institutes of Health (NIH) https://www.covid19treatmentguidelines.nih.gov/overview/clinical-spectrum/. Study participants were classified according to i) blood pressure (mmHg) values into normotensive (NT) and hypertensive (HT); ii) body mass index (BMI) into lean or normal weight (NW) (BMI <25 kg/m^2), overweight (OW) (25≤BMI<30 kg/m^2), and obese (OB) (BMI ≥30 kg/m^2); and iii) COVID-19 severity into asymptomatic or mild, moderate (Mod), severe disease and critical (SV). Based on our assessment of the responders' answers to electronically published survey between July and September of 2021, individuals who fulfilled the study criteria and gave Informed consents to participate were face–to-face interviewed to review their PCR and other lab results and chest X-ray image reports. Three milliliters of peripheral blood were collected on Ethylene Diamine Tetra-acetic Acid (EDTA) and stored at -20 C° at Damascus University Blood Center. The majority of the participants (n=T39 subjects, 72.22%) were NT whereas 15 were HT (27.78%). Twenty subjects (37.04%) were lean, 19 (35.19%) were overweight (OW), and 15 (27.78%) were obese (OB). Twenty (37.04%) subjects were asymptomatic or with mild symptoms, 18 (33.33%) were moderate cases (with SpO2 ≥94%), and 16 (29.63%) were severe cases with SpO2 <94% or life threatening conditions. Our study was approved by both the Bioethics Committee at the Faculty of Pharmacy- Damascus University, and the National Committee for Ethics of Scientific Research and Novel Technologies (CONEST), affiliated with the Higher Commission for Scientific Research. Informed consents were obtained from all subjects prior to their enrollment.

DNA Isolation and Amplification With the exception of DNA sequencing, the molecular work and electrophoresis-based genotyping processes were performed at the Pharmaceutical Biotechnology and Immunology laboratories- National Commission for Biotechnology, Damascus. Genomic DNA was isolated from blood samples using Qiagen blood DNA extraction kit (Qiagen, USA) according to the manufacturer's protocol. DNA concentrations and purity were assessed using Nano drop (Maestrogen®, Taiwan). In order to verify the quality and conservation of isolated DNA, horizontal 1.5% agarose gel electrophoresis was run followed by ethidium bromide staining (Promega®, USA).

We performed polymerase chain reaction (PCR) amplification using two sets of specific primer pairs. For the rs1799752 (ACE I/D), we used forward primer 5` CTGGAGAGCCACT CCCATCCTTTCT 3` and reverse primer 5` GGG ACGTGGCCATCACATTCGTCAG 3`, with expected amplicon sizes of 190 bp for the D allele and 490 bp for the I allele. For the AGT rs699, we used forward primer 5` GTGGTCAC CAGGTATGTCCG 3` and reverse primer 5` TATACCCCTGTGGTCCTCCC 3`, with an expected amplicon size of 291 base pair (bp). All primers were manufactured by Eurofins (Belgium), and were designed using Primer 3 software tool and checked prior to ordering using the MFE primer bioinformatics tool (https://mfeprimer3.-igenetech.com/spec) to evade any non-specific binding to genomic DNA. PCR was performed according to standard methods using thermal cycler (SENSEQUEST®, Germany) and 2X Master Mix (Genedirex®, Taiwan). The PCR reaction mixture contained 20 ng of genomic DNA and 0.25 µM/l of each primer. PCR amplification was performed according the following protocol including: initial denaturation for 5 min at 95° C, 35 cycles of (30 sec at 94° C, 30 sec at 57° C, and 1 min at 72° C), and a final 10 min at 72° C for final extension. PCR amplicons were analyzed by agarose gel electrophoresis, using an electrophoresis apparatus from (Cleaver, UK) and a 100 bp DNA size marker from (Genedirex®, Taiwan). DNA Sequencing was performed for the AGT rs699 specific amolicons at

Macrogen® (South Korea) according to standard protocols.

Bioinformatics and Statistical Analyses

Sequencing results were analyzed using bioinformatics tool (Geneious® software, USA). Genotype frequencies of sample alleles were estimated by gene counting method. The agreement with Hardy-Weinberg equilibrium (HWE) of the observed genotypic distribution, and comparisons of the frequencies of *the AGT* and *ACE* genes alleles and genotypes were tested by chi-square tests using chi-square calculator at https://www.socscistatistics.com/tests/. Finally, EXCEL t-test calculator was used to compare means of age and BMI among subgroups. For all statistical tests, a p-value was set at <0.05 for statistical significance.

Results:

Genotyping

Amplification of *AGT and ACE* gene target sequences was successful, with amplicons appeared with the expected sizes, between 200 and 300 bp for AGT, and either of ~200 bp amplicon for homozygosity of the deletion allele, ~500 bp amplicon for homozygosity of the insertion allele, or both amplicons for heterozygousity of the ACE INDEL (Fig. 1 a and b). For the AGT rs699, single nucleotide polymorphisms were evident from reading the sequencing chromatograms with either GG (or CC) homozygote, AA (or TT) homozygote, or AG (or TC) heterozygote genotypes (Fig 1 c).

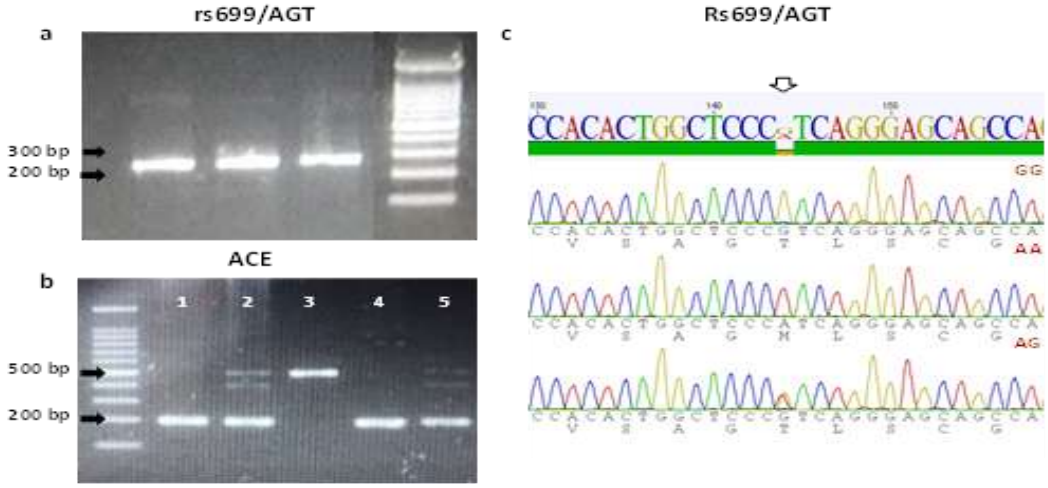

Figure (1): Amplification and genotyping of AGT and ACE specific amplicons containing the rs699 and rs1799752. A) A representative gel electrophoresis of three AGT rs699 specific PCR amplicons (291 bp). b) Amplicons of ACE I/D showing either a single band at 190 bp (the DD genotype) (lanes 1&4) or at 490 bp (the II genotype) (lane 3), or both bands (the heterozygous I/D genotype) (lanes 2 & 5). c) Representative sequencing chromatograms for AGT rs699 showing either the GG (CC) or AA (TT) homozygotes, or the AG (TC) heterozygotes.

Allele Frequencies

We compared allele frequencies for both AGT rs699 and ACE indel polymorphisms on the basis of gender, blood pressure, BMI values, and severity of COVID-19 disease (Table 1). As shown in Table 1, no differences in allele frequencies were observed between females versus males, normotensive versus hypertensive patients, nor between patients with different COVID-19 severity ($p > 0.05$), although both hypertensive and severe COVID-19 patients showed relatively high allele C frequencies (63.3%) and (65.6%), respectively. Nevertheless, there was a significant difference in the frequency of the ACE rs1799752 (I) allele between lean (NW) and overweight OW patients (20% versus 44.7%, $p = 0.019$) and between lean and both overweight OW and obese OB patients together (20% versus 41.2%, $p = 0.024$), respectively.

Table (1): Allele frequencies for the AGT rs699 and ACE rs1799752 in the study subjects classified based on gender, blood pressure, obesity, and COVID-19 severity.

Category (No. Subjects)	AGT rs699 No. Alleles (% Frequency)		P values	ACE rs1799752 No. Alleles (% Frequency)		P values
	T	C		I	D	
Gender						
Females (24)	25 (52.1)	23 (49.6)	0.365	16 (33.3)	32 (66.7)	1
Males (30)	26 (43.3)	34 (56.7)		20 (33.3)	40 (66.7)	
Blood Pressure						
Normotensive (39)	40 (51.3)	38 (48.7)	0.173	25 (32.1)	53 (67.9)	0.655
Hypertensive (15)	11 (36.7)	19 (63.3)		11 (36.7)	19 (66.3)	
BMI						
NW (20)	21 (52.5)	19 (47.5)	-----	8 (20)	32 (80)	-----
Overweight (OW) (19)	17 (44.7)	21 (55.3)	0.492[a]	17 (44.7)	21 (55.3)	**0.019[a]***
Obese (OB) (15)	30 (43.3)	13 (56.7)	0.106[a]	11 (36.7)	19 (63.3)	0.120[a]
OW + OB (34)	30 (44.1)	38 (55.9)	0.399[a]	28 (41.2)	40 (58.8)	**0.024[a]***
BMI <50 yrs						
NW <50 yrs (18)	20 (55.6)	16 (44.4)	0.474	5 (13.9)	31 (86.1)	**0.0012***
OW and OB <50 yrs (16)	15 (46.9)	17 (53.1)		16 (50)	16 (50)	
COVID-19 Severity						
Mild (20)	20 (50)	20 (50)	-----	13 (32.5)	27 (67.5)	-----
Moderate (Mod) (18)	20 (55.6)	16 (44.4)	0.628[b]	11 (30.6)	25 (69.4)	0.855[b]
Severe (SV) (16)	11 (34.4)	21 (65.6)	0.183[b]	12 (37.5)	20 (62.5)	0.657[b]
Mod + SV (34)	31 (45.6)	37 (54.4)	0.657[b]	23 (33.8)	45 (66.2)	0.879[b]

[a]: p-values of chi–test for comparisons with lean (NW) subjects. [b]: p-values of chi–test for comparisons with mild disease subjects *****: Statistically significant difference ($p<0.05$). BMI: body mass index.

Furthermore, the allele I frequencies was about 2.5 fold higher in young (<50 years old) OW+OB compared to their counterpart lean subjects, p = 0.0012. In fact, our data support an evident association between both age and obesity with COVID-19 severity. Most patients with severe COVID-19 disease were either overweight or obese (13 out of 16, 81.3%) in addition to being mostly over 50 years old (12 out of 16, 75%). The association between age with COVID-19 severity is illustrated in Fig 2 a, where it clearly demonstrates a significantly higher mean of age (58.7 ± 17.5 years, mean ± standard deviation) in patients who suffered severe COVID-19 compared with those of patients with either moderate or mild symptoms (38 ± 14.5 years, and 37.2 ± 12.4 years, p=0.0001 and p=0.0007, respectively). The data also prove an association between BMI and age, as obese and overweight patients were
significantly older (52.8 ± 15.7 and 47.1 ± 19.2 years, respectively) in comparison to the lean (34.1 ± 11.8 years) (p=0.015 and p= 0.0003, respectively). Patients with hypertension had significantly older mean of age (62.2 ± 13.3 years) compared to normotensive (36.8 ± 13.2 years) patients ($p=5.6 \times 10^{-8}$). Finally, no significant difference in age was noted between females (40.3 ± 17.2 years) versus males (46.7 ± 17.4 years) (p=0.18) (Fig 2 a).

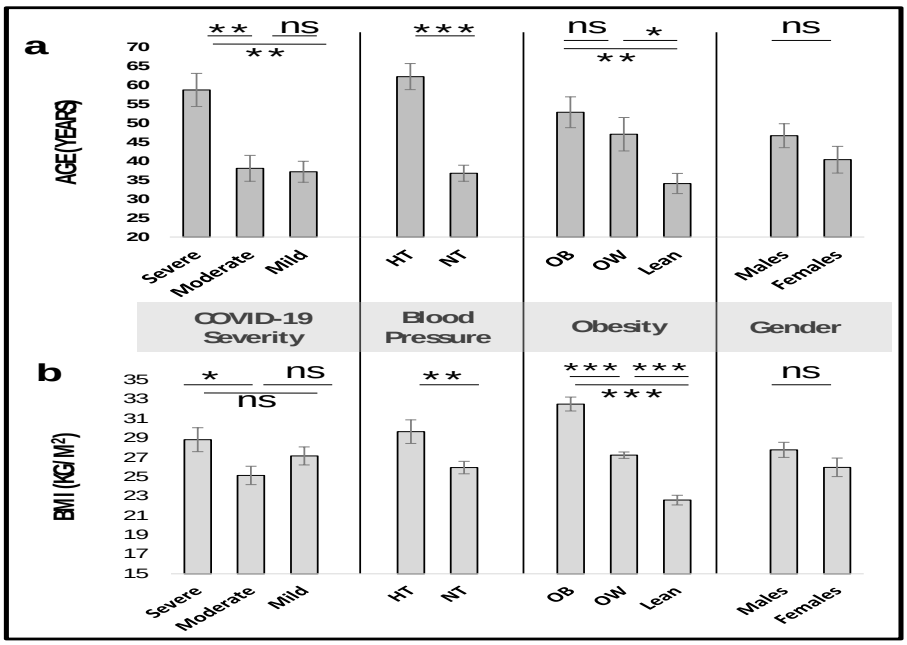

Figure (2): Bar graphs presenting means of age (a) and BMI (b) of participants categorized according to gender, BMI, blood pressure, and COVID-19 severity. ns: not significant, * p < 0.05 - ** p < 0.001 - *** p < 0.00001.

Similarly, an association between obesity and COVID-19 severity was observed (Fig 2 b). In this context, patients with severe COVID-19 symptoms had a significantly higher BMI (28.8 ± 4.9 kg/m2) compared to patients with moderate symptoms (25.1 ± 4.02 kg/m2), (p=0.022), but not with patients with mild symptoms (27.1 ± 4.2 kg/m2) (p=0.270). Furthermore, a significantly higher BMI was noticeable in hypertensive (29.6 ± 4.8 kg/m^2).

We next examined allele frequencies of both the rs699 and rs1799752 polymorphisms in COVID-19 patients taking into consideration the abovementioned independent variables (i.e., obesity and age). To achieve this goal, we calculated allele frequencies in mild, moderate and severe COVID-19 patients sorted firstly, according to BMI, i.e., <25 Kg/m^2 or ≥25 Kg/m^2, and secondly, based on age categories (<50 years and ≥50 years). Data presented in Table 2 show that the frequencies of the rs699 C and rs1799752 I alleles were higher in patients with BMI ≥25 kg/m^2 compared to lean patients (BMI<25 kg/m^2), but these differences lack any statistical significance (p >0.05 for all comparisons). Nevertheless, the only proved significant difference was for the rs1799752 I and D allele frequencies in patients with moderate COVID-19 disease whose BMI ≥25 k/m2 (50% I allele and 50% D allele) compared to patients with BMI <25 kg/m2 (15% I allele and 85% D allele) (p=0.023). We recorded no significant differences in either rs699 or rs1799752 allelic frequencies when patients were classified according to age, i.e., <50 years and ≥50 years, although the frequency of the I allele almost doubled in older patients (≥50 years) with mild and moderate COVID-19 disease compared to their younger peers (<50 years).

Genotype Frequencies

We compared frequencies for the rs699 genotypes (TT-TC-CC) and rs1799752 genotypes (II-ID-DD) among study subgroups classified according to gender, blood pressure, BMI, and COVID-19 disease severity (Table 3).

AGT rs699 (T>C)

We recorded a significantly higher frequency of the rs699 CC genotype in males (40%) compared to females (12.5%), with lower TC frequency in males (33.3%) compared to females (70.8%) (p=0.0185). In hypertensive patients, the CC genotype frequency was almost double (40%) compared to normotensive patients (23.1%) but with no statistically significant

difference (p=0.386). Additionally, no differences were detected in CC or TC genotype frequencies when classifying patients according to BMI, despite a close to a double CC frequency in young (>50 years old) overweight and obese patients compared to age matched lean patients (31.2% versus 16.7%, respectively), p=0.59. Lastly, we recorded no significant differences between the rs699 genotype frequencies in association with COVID-19 severity, although the highest CC genotype frequency was recorded in patients with severe disease (37.5%) compared with patients with mild COVID-19 (30%), p=0.19 (Table 3).

Table (2): Allele frequencies for the AGT rs699 or ACE rs1799752 in the study subjects classified based on COVID-19 severity after adjusting for either BMI or age.

Category (No. Subjects)	AGT rs699 No. Alleles (% Frequency)		P values	ACE rs1799752 No. Alleles (% Frequency)		P values
	T	C		I	D	
COVID-19 Severity/BMI (kg/m2)						
Mild, BMI <25 (7)	8 (57.1)	6 (42.9)	0.507	2 (14.3)	12 (85.7)	0.071
Mild, BMI ≥25 (13)	12 (46.2)	14 (53.8)		11 (42.3)	15 (57.7)	
Moderate, BMI < 25 (10)	11 (55)	9 (45)	0.940	3 (15)	17 (85)	**0.023***
Moderate, BMI ≥25 (8)	9 (56.3)	7 (43.7)		8 (50)	8 (50)	
Mod + SV, BMI < 25 (13)	13 (50)	13 (50)	0.565	6 (23.1)	20 (76.9)	0.140
Mod + SV, BMI ≥25 (21)	18 (42.9)	24 (57.1)		17 (40.5)	25 (59.5)	
COVID-19/Age						
Mild, Age < 50 yrs (16)	18 (56.3)	14 (43.7)	0.113	10 (31.2)	22 (68.8)	0.102
Mild, Age ≥50 yrs (4)	2 (25)	6 (75)		5 (62.5)	3 (37.5)	
Moderate, Age < 50 yrs (14)	15 (53.6)	13 (46.4)	0.654	7 (25)	21 (75)	0.175
Moderate, Age ≥50 yrs (4)	5 (62.5)	3 (37.5)		4 (50)	4 (50)	
Mod + SV, Age < 50 yrs (18)	17 (47.2)	19 (52.8)	0.774	11 (30.6)	25 (69.4)	0.545
Mod + SV, Age ≥50 yrs (16)	14 (43.8)	18 (56.2)		12 (37.5)	20 (62.5)	
Mod: moderate. SV: severe. *: Statistically significant difference (p<0.05).						

Table (3): Genotype frequencies for AGT rs699 or ACE rs1799752 in the study subjects classified based on gender, blood pressure, obesity, and COVID-19 severity.

Category (No. Subjects)	AGT rs699 No. Genotypes (% Frequency)			P values	ACE rs1799752 No. Genotypes (% Frequency)			P values
	TT	TC	CC		II	ID	DD	
Gender								
Females (24)	4 (16.7)	17 (70.8)	3 (12.5)	**0.0185***	2 (8.3)	12 (50)	10 (41.7)	0.713
Males (30)	8 (26.7)	10 (33.3)	12 (40)		4 (13.3)	12 (40)	14 (46.7)	
Blood Pressure								
Normotensive (39)	10 (25.6)	20 (51.3)	9 (23.1)	0.386	4 (10.3)	17 (43.6)	18 (46.1)	0.901
Hypertensive (15)	2 (13.3)	7 (46.7)	6 (40)		2 (13.3)	7 (46.7)	6 (40)	
BMI (kg/m2)								
NW (20)	5 (25)	11 (55)	4 (20)	-----	1 (5)	6 (30)	13 (65)	-----
Overweight (OW) (19)	3 (15.8)	11 (57.9)	5 (26.3)	0.746[a]	4 (21)	9 (47.4)	6 (31.6)	0.083[a]
Obese (OB) (15)	4 (26.7)	5 (33.3)	6 (40)	0.351[a]	1 (6.7)	9 (60)	5 (33.3)	0.172[a]
OW + OB (34)	7 (20.6)	16 (47)	11 (32.4)	0.618[a]	5 (14.7)	18 (52.9)	11 (32.4)	0.061[a]
BMI <50 yrs								
NW <50 yrs (18)	5 (27.8)	10 (55.5)	3 (16.7)	0.598	0 (0)	5 (27.8)	13 (72.2)	**0.012***
OW and OB <50 yrs (16)	4 (25)	7 (43.8)	5 (31.2)		3 (18.75)	10 (62.5)	3 (18.75)	
COVID-19 Severity								
Mild (20)	6 (30)	8 (40)	6 (30)	-----	3 (15)	7 (35)	10 (50)	-----
Moderate (Mod) (18)	5 (27.8)	10 (55.5)	3 (16.7)	0.545[b]	1 (5.5)	9 (50)	8 (44.5)	0.503[b]
Severe (SV) (16)	1 (6.3)	9 (56.2)	6 (37.5)	0.199[b]	2 (12.5)	8 (50)	6 (37.5)	0.659[b]
Mod + SV (34)	6 (17.6)	19 (55.9)	9 (26.5)	0.459[b]	3 (8.8)	17 (50)	14 (41.2)	**0.040[b]***

[a]: p-values of chi–test for comparisons with lean subjects. [b]: p-values of chi–test for comparisons with mild disease subjects. *: Statistically significant difference (p<0.05). BMI: body mass index.

The ACE rs1799752 (D>I)

The frequencies of II, ID, and DD genotypes were comparable in both genders and also between normotensive and hypertensive patients. On the contrary, our data show considerably higher ID genotype frequencies in overweight and obese subjects compared to lean patients, with chi square p values slightly higher than 0.05 (p=0.061) (Table 3). Nevertheless, when including younger patients (<50 years old) in all groups, the frequency of the ID genotype was more than two folds higher in overweight and obese (62.5%) compared to lean (27.8%) patients, while the frequency of DD genotype dropped from 72.2% in lean to only 18.75% in overweight and obese patients (p= 0.012). Furthermore, our data showed a higher frequency of the ID genotype in patients with moderate and severe COVID-19 disease (50%) compared to lower frequency (35%) in patients with mild symptoms (p=0.04).

The majority of the AGT rs699 and ACE rs1799752 genotype distributions among different categories were in agreement with HWE (as shown in Table 3). Whereas the ACE rs1799752 genotype frequencies were in accordance to HWE among both females and males, rs699 genotype frequencies were not. Additionally, the distributions of the rs699 genotypes (TT 26.7%, TC 33.3%, and CC 40%) and rs1799752 genotypes in obese patients (II 6.7%, ID 60%, and DD 33.3%) both deviated from HWE

(p=0.0012 and p=0.0035, respectively). Other genotype frequency deviations from HWE encompass the rs1799752 genotypes in overweight and obese young (<50 years old) patients, in both rs699 (TT 30%, TC 40%, and CC 30%) and rs1799752 genotypes (II 15%, ID 35%, and DD 50%) in patients with mild COVID-19, and finally in the rs699 genotypes in patients with severe COVID-19 symptoms (TT 6.3%, TC 56.2%, and CC 37.5%).

Haplotype Classification

As for haplotype classification, we counted the frequencies of the possible nine haplotypes (3^2) in the total 54 subjects enrolled in the study, classified according to COVID-19 severity into mild or moderate and severe disease (Table 4). Our results demonstrate highest haplotype frequencies of the CC-DD (5/20 subjects, 25%) followed by TC-DD or TT-ID (four subjects each, 20%) in the subgroup of subjects with mild disease, whereas the TC-ID (11/34 subjects, 32.4%), TC-DD (7/34 subjects, 20.6), and CC-DD (4/34 subjects, 11.8%) constituted the highest haplotype frequencies in subjects with moderate and severe disease. Consistent with the findings presented in Figure 2, Table 4 also demonstrates older age mean in subjects with moderate and severe COVID-19 in comparison with those with mild disease.

Table (4): Frequencies of the nine haplotypes among the study subjects sorted/ according to COVID-19 severity (mild disease versus moderate and severe disease).

	Haplotypes (No. subjects)								
Disease Severity	**TT-II (1)**	**TT-ID (7)**	**TT-DD (4)**	**TC-II (3)**	**TC-ID (13)**	**TC-DD (11)**	**CC-II (2)**	**CC-ID (4)**	**CC-DD (9)**
Mild (20)	**1**	**4**	**1**	**2**	**2**	**4**	**--**	**1**	**5**
Age (yrs.)	31	29.8	25	47	39	36.3	--	29	44.6
BMI (kg/m^2)	29	27.3	22	26.6	27.8	24.7	--	32	28.8
M + S (34)	**--**	**3**	**3**	**1**	**11**	**7**	**2**	**3**	**4**
Age (yrs.)	--	36.7	47.7	49	47.6	45	65	46	54.3
BMI (kg/m^2)	--	24.5	28.6	29	29.3	22.8	26.5	28	26.8
M: moderate. S: severe. BMI: body mass index.									

Discussion:

In this study, we aimed to identify possible association(s) between COVID-19 severity and two genetic variants in the previously well characterized *AGT* and *ACE* genes, encoding proteins linked unquestionably to the RAAS system and possibly to other morbidities, including hypertension and obesity. The RAAS axis is known to regulate fluid balance, blood pressure and cardio-renal function [13]. Previously, several lines of evidence supported the association of the RAAS system with obesity, hypertension and COVID-19 disease severity.

To proceed with the goals of our study, we interviewed 54 individuals with well-established diagnosis of COVID-19 with a wide spectrum of disease severity. Genotyping of the study subjects was performed to examine the proposed association between two polymorphisms, namely the rs699 in *AGT* and rs1799752 in *ACE*, with obesity, hypertension and COVID-19 severity. We then classified the study participants according to their gender, obesity, blood pressure status, and COVID-19 severity, and reported the allelic and genotypic frequencies of the two studied polymorphisms. In the following paragraphs, we will discuss our results according to the study subject subgroups.

Gender and Age

Several previous reports demonstrated a more severe COVID-19 illness in males compared with females [14-16]**,** and that male gender is an independent risk factor for COVID-19 severity [17]. Our data demonstrate no differences in age or BMI between males and females. Moreover, we found no differences in the rs699 and rs1799752 allelic frequencies between male and female individuals (Table 1). However, we report a significantly higher frequency of the rs699 CC genotype while lower frequency of TC in males compared to females (40%

versus 12.5%, for CC genotype and 33.3% versus 70.8% for TC, respectively) (Table 3). On the contrary, the frequencies of all three rs1799752 genotypes were closely comparable between males and females. This finding is in agreement with the results from a previous study that reported a significantly higher rs699 TT genotype in Indian females compared with males, whereas no difference was observed in regards to the rs1799752 genotype frequencies, [18], in contrary to AL-Eitan et al who reported a significantly higher I/D frequency in Jordanian males compared to females patients [19].

It is noteworthy that the genotype frequencies in females and males were in accordance with HWE only for rs1799752 but not for rs699. This deviation from normal genotype distribution in the rs699 may emphasize the possible bias in the COVID-19 patient population, and therefore might explain the overrepresentation of male patients with the CC genotype in our patient population compared to either female patients or the general population. Although the explanation for these findings is rather obscure, we could postulate that the TC genotype is a risk factor especially in female subjects. In fact, Repchuk et al reported an association between the rs699 and obesity and essential arterial hypertension but only in females [20]. In a recent report however, the frequency of the CC genotype was only 23% in healthy Egyptians, while the distribution of the rs699 genotypes was in accordance with HWE [21]. It remains to investigate the frequencies of CC and TC genotypes in the normal Syrian population.

On the other hand, our data clearly demonstrate that age was an independent factor correlated with obesity, hypertension and COVID-19 severity. This was evident by the fact that older subjects (≥50 yrs. old) were overrepresented in all obese, hypertensive and severe COVID-19 groups (Fig 2a). Our data are in accordance with several previous reports that showed significantly higher mean age in patients with severe COVID-19 disease [14,22]. However, taking into account the association of age with many other factors, it is rather impossible to isolate the contribution of age from those of other factors.

Blood Pressure

Despite the absence of a statistically significant difference in the rs699 allelic frequencies between normotensive (NT) and hypertensive (HT) individuals, the frequency of the C allele was considerably higher in HT compared with NT subjects, while very comparable rs1799752 I and D allele frequencies were found in NT and HT individuals (Table 1). Moreover, HT individuals had a considerably higher frequency of the rs699 CC genotype compared with NT individuals, while the frequencies of the rs1799752 genotypes were comparable between NT versus HT subjects (Table 3). Indeed, the link between the rs699 T or C alleles with Hypertension is still controversial. Two previous reports demonstrated association between the C allele or CC genotype with essential hypertension [23,24], while another report suggested that the CC genotype was protective and reduced the relative risk almost by half for high rates of BP elevation in patients with essential hypertension [20]. Finally, Fornage et al found no statistical association between the rs699 polymorphism and the occurrence of essential hypertension [25]. Hence, it seems that the involvement of the rs699 polymorphism in increasing hypertension might be affected by yet not well-identified confounding factors related to different study designs, inclusion criteria, or ethnicities. Relevantly, our data showed that HT individuals were significantly older and more obese compared with NT subjects (Fig 2 a,b). This finding well aligned with previous reports documenting that both age and obesity are independent risk factors for hypertension [13,14,26-28], making the isolation of the C allele or the CC genotype effect on hypertension rather intricate.

Obesity

As demonstrated in Fig 2b, obese subjects constituted a considerably high percentage in both hypertensive and severely ill COVID-19 groups. On the other hand, a strong evidence for an association between genetic makeup and obesity was provided; as a clear discrepancy exist in the I and D allele distribution between lean/normal weight (NW) subjects and their overweight/obese counterparts. Our findings demonstrate a higher rs1799752 I allele frequency and lower D allele frequency in overweight (OW) and obese (OB) individuals compared to lean subjects (Table 1), although this difference was

statistically significant only when comparing the I allele frequencies in either OW (44.7%) or OW plus OB together (41.2%) with that in lean subjects (20%). The significant difference in allele I frequency was more profound in young (<50 year old) subjects between OW plus Ob (50%) compared to lean (13.9%). Moreover, the sum of frequencies of II and ID genotypes were higher (68.4%) in OW, OB (66.7%), and OW plus OB (67.6%) in comparison with lean subjects (35%) (Table 3). Nevertheless, this difference reached statistical significance only in younger (<50 years old) subjects in all three groups, where the frequencies of II and ID genotypes collectively were higher in OW plus OB (II 18.75% + ID 62.5% = 81.25%) compared to (II 0% + ID 27.8% = 27.8) in lean subjects (p=0.012) (Table 3). Altogether, our results prove overrepresentation of the I allele in obese individuals and therefore are in agreement with results reported by Kwon in Korean subjects [29]. Nevertheless, our data disagree with several lines of evidence supporting a dominant dogma of an association of the D allele with obesity, as reported in Egyptian children [30], Chinese subjects [31] and Tunisian subjects [32]. Nevertheless, some other reports however failed to provide evidence on any significant association between the rs1799752 polymorphism and obesity in Malay subjects [33].

As for allelic frequencies of the rs699 polymorphism, our data do not support any significant association between either T or C alleles, nor between TT, TC, or CC genotypes with increased BMI, as no significant differences were observed in allele or genotype frequencies in OW and OB subjects in comparison with lean subjects (Tables 1 and 3). Of note however, the highest C allele frequency (56.7%) and CC genotype frequency (40%) were found in OB subject. Indeed, obesity is rather a complex disease that results, among many other abnormalities, in dysregulated adipose tissues, which is associated with angiotensinogen secretion and enhancement of the RAAS [13,34]. Hence, this adds levels of complexity that might obscure the detection of a genetic effect, such as the investigated polymorphisms.

COVID-19 Severity

Study subjects were classified according to the severity of COVID-19 disease into three categories; mild, moderate and severe disease. As for allele frequencies for both the rs699 and rs1799752 polymorphisms, we found no significant differences in allele frequencies between the subjects with moderate or severe disease in comparison with those with mild symptoms (Table 1). Nevertheless, the highest frequencies of the rs699 C allele (65.6%) and rs1799752 I allele (37.5%) were recorded in subjects with severe COVID-19 cases. To adjust for either obesity or age possible association with COVID-19 severity, we reclassified the study subjects according to BMI (i.e., <25 versus ≥25 kg/m2), or according to age (i.e., <50 versus ≥50 years old) (Table 2). We found no significant differences between all reclassified groups except for the statistically significant higher frequency of the rs1799752 I allele in subjects with moderate COVID-19 with BMI ≥25 (50%) compared with those with BMI <25 (15%). Furthermore, no significant differences were observed when comparing frequencies of all three rs699 genotypes between subjects with either moderate or severe disease and those with mild disease (Table 3). Of note however, subjects with moderate and severe COVID-19 had higher frequency of the TC genotype compared with those with mild disease. Though, our data demonstrated a significant difference in the rs1799752 genotype frequencies between subjects with moderate and severe disease (II 8.8%, ID 50%, and DD 41.2%) in comparison with those with mild disease (II 15%, ID 35%, and DD 50%) (p=0.04).

Indeed, the association between either rs699 or rs1799752 polymorphism with COVID-19 severity is apparent for the rs699 compared with rs1799752, whose involvement in rather controversial. Kouhpayeh et al found that the C allele of AGT rs699 and the T/C genotype were associated with increased the risk of COVID-19 infection [22]. In addition, Cafiero et al. found that asymptomatic patients had a lower frequency of the T/C genotype compared to symptomatic patients with COVID-19 [35]. Of note, the T/C genotype was recently found to be associated with increased risk for cardiovascular disease [36]. As for the rs1799752

Indel polymorphism, several reports suggested an association between the D allele and DD genotype with COVID-19 severity, and a protective role for the I allele [19,37-41]. Conversely, Çelik et al and Mart´ınez-Go´ mez et al found no association between the I/D genotype and COVID-19 severity [17,42]. In agreement with our findings, Faridzadeh et al reported a worse prognosis for Iranian patients with the II + ID genotypes compared with those with the DD genotype [43]. Lastly, Saad et al found a positive association between the I allele and the risk of contracting the COVID-19 disease, while a positive association between the D allele and a worse outcome of the COVID-19 infection [44]. Our data are in line with the findings reported by Cafiero et al [35] of a higher TC genotype frequency in patients with moderate and severe COVID-19 compared to mild disease. Furthermore, the results of Faridzadeh et al [43] provide strong support to our conclusions of associations of the I allele and the I/D genotype with COVID-19 severity (Tables 2 and 3).

Additionally, when comparing distribution of all possible nine haplotypes of the rs699 and rs1799752 according to subgroups, we found that eleven of the 34 subjects (32.35%) with moderate and severe disease genotyped as [TC-ID] in comparison with only two of 20 individuals (10.00%) with [TC-ID] genotype in the mild

disease group. In contrast, subjects with mild conditions were mainly carriers of the CC-DD (25% of subjects) compared to only (11.8% of subjects) in the moderate and severe group (Table 4). This could again link either rs699 TC or rs1799752 ID or both genotypes to increased COVID-19 severity. Nevertheless, due to the small size of our cohort, the effect(s) of age and obesity cannot be excluded, as both are possibly linked to disease severity.

Conclusions:

In this report, we identified the allele, genotype, and haplotype frequencies of two gene polymorphisms in *AGT* and *ACE genes*, encoding for two key proteins in the RAAS axis. In a cohort of Syrians previously diagnosed with COVID-19, our data suggest an association between the rs1799752 I allele with obesity, and a more evident association in younger (<50 years) subjects. Furthermore, the frequency of the rs699 CC genotype was higher whereas that of the TC genotype was lower in male compared to female patients. Although the explanation for this finding remains unintelligible, we could postulate that the TC genotype is a risk factor in female subjects, supported by the fact that the distribution of the rs699 did not follow HWE, making this genotype a specific marker for severity in female COVID-19 patients. Finally, we found that both rs699 TC and rs1799752 ID genotypes, and the TC-ID haplotype, were positively associated with COVID-19 severity in our cohort.

Since the main limitation of our study is the small number of recruited subjects, further research could be done in the future to confirm the involvement of the two AGT and ACE polymorphisms in obesity and COVID-19 severity in a larger cohort.

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