Type 1 diabetes is associated with significant changes of ACE and ACE2 expression in peripheral blood mononuclear cells

Federica Tonon [a], Riccardo Candido [b], Barbara Toffoli [a], Elisabetta Tommasi [b], Thomas Cortello [a], Bruno Fabris [a,c], Stella Bernardi [a,c,*]

[a] Department of Medical Surgical and Health Sciences, University of Trieste, Trieste, Italy
[b] Centro Diabetologico Distretto 3 e 4, Azienda Sanitaria Universitaria Giuliano-Isontina, Trieste, Italy
[c] SS Endocrinologia, UCO Medicina Clinica, Ospedale di Cattinara, Azienda Sanitaria Universitaria Giuliano-Isontina, Trieste, Italy

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Abstract *Background and aims:* The renin-angiotensin system (RAS), which is a key mediator of cardiovascular homeostasis, has two main axes. The classic one, including angiotensin-converting enzyme (ACE) and Angiotensin (Ang) II, promoting vasoconstriction, and the "alternative" one, including ACE2 and Ang1-7, with opposed actions to AngII. ACE2 has been identified as the main receptor of SARS-CoV2, whereby it enters the cells, leading to the downregulation of surface ACE2 and RAS tissue unbalance. Given that diabetes is associated with an increase in COVID-19 severity and death, we aimed at evaluating RAS expression in patients with type 1 diabetes (T1D).

Methods and results: This is a case–control study comparing 39 T1D patients to 33 controls, with a median age of 29 and 32 years, and no comorbidities. ACE and ACE2 gene expression was assessed in peripheral blood mononuclear cells. T1D patients had higher ACE expression and circulating AngII, which were related to glucose levels. T1D patients had lower ACE2 expression. However, ACE2 expression was also related to the sex of participants, being higher in the female group. T1D women did not show the same increase of ACE2 expression that was seen in control women.

Conclusion: T1D promotes the increase of ACE, AngII, and ACE/ACE2, which might contribute to the higher cardiovascular risk, as well as to severe tissue injury induced by SARS-CoV2 in these patients. The ratio ACE/ACE2 does not differ between men and women with T1D, which might explain why CVD or COVID-19 do not show substantial gender differences in these patients.

1. Introduction

The year 2020 will be remembered for the deadly outbreak of coronavirus disease 19 (COVID-19), a pandemic due to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV2). Looking at the susceptibility and natural history of SARS-CoV2 infection in patients with diabetes, diabetes has not been associated with increased susceptibility to COVID-19 [1], but the infection resulted in

* Corresponding author. Department of Medical Surgical and Health Sciences, University of Trieste, Cattinara Teaching Hospital Strada di Fiume, 34149, Trieste, Italy. Fax: +0039 0403994246.
E-mail addresses: stella.bernardi@asugi.sanita.fvg.it, shiningstella@gmail.com (S. Bernardi).

The Italian Diabetes Society, the Italian Society for the Study of Atherosclerosis, the Italian Society of Human Nutrition and the Department of Clinical Medicine and Surgery, Federico II University.

increased rates of hospitalization and greater severity of illness in patients with both type 1 and type 2 diabetes. Barron et al. demonstrated that people with diabetes were at higher risk of COVID-19-related mortality, and that this was independent of age, sex, deprivation, ethnicity, geographical region, as well as cardiovascular comorbidities [2]. Also Lampasona et al. reported that diabetes was independently associated with risk of death for COVID-19, even after adjustment for age, sex and other relevant comorbidities [3]. Moreover, in that work, the Authors found a strong association between higher glucose levels and risk of death, irrespective of diabetes diagnosis [3]. Nevertheless, the mechanisms underlying enhanced COVID-19 severity and mortality in patients with diabetes remain uncertain [1].

Angiotensin-converting enzyme 2 (ACE2) has been identified as the main SARS-CoV and SARS-CoV2 receptor [4,5]. ACE2 is a transmembrane protein that is expressed throughout the body, including the lungs, vessels, heart, intestine, central nervous system, kidney, and liver [6]. The cellular entry of SARS-CoV2 is due to the interaction between the viral S-protein and ACE2 extracellular domains [4], followed by subsequent downregulation of surface ACE2 expression [7,8]. The interaction between SARS-CoV2 and ACE2 in COVID-19 pathophysiology is key not only to the susceptibility to the disease, which theoretically should be associated to the amount of tissue ACE2, but also to the natural history of the disease, which worsens with ACE2 loss. Experimental studies have shown that ACE2 protects from severe acute lung failure [8,9].

ACE2 is one of the main regulators of the renin angiotensin system (RAS). The discovery of ACE2, which dates back to the year 2000 [6,10], has changed our understanding of the RAS, which is now regarded as a system made of two main axes with opposed actions: the vasoconstrictor, pro-infammatory ACE/AngII/AT1R axis and the "new" vasodilating anti-inflammatory ACE2/Ang1-7/MasR axis [11], as shown in Fig. 1. In this setting, ACE2 is the enzyme that degrades the vasoconstrictor and pro-inflammatory AngII into Ang 1–7, which is a peptide with opposite actions to those of Ang II. Consequently, it is the ratio ACE/ACE2 to determine the final circulating and tissue levels of AngII [12,13]. Consistent with this, ACE2-deficiency leads to an overexpression of the ACE/AngII/AT1R axis and an imbalance of RAS, which might favor local inflammation, tissue damage [14] and the clinical severity of SARS-CoV2.

Based on this background, we aimed to evaluate the expression of RAS components in patients with type 1 diabetes under normal conditions/circumstances.

2. Methods

2.1. Study design

This is an observational case–control study, aimed to evaluate the expression of renin-angiotensin components in patients with type 1 diabetes mellitus (T1D). Patients with diagnosis of T1D, aged between 20 and 45 years, were recruited at the Diabetes Center District 3 (ASUGI), while age- and sex-matched healthy volunteers (controls) were recruited at the Insitute of Medicina Clinica (ASUGI). Recruitment was performed with the use of electronic medical records. In both groups, exclusion criteria were: (*i*) intercurrent acute disease; (*ii*) BMI >30 kg/m^2; (*iii*) use of drugs interfering with the renin-angiotensin system (ACE inhibitors, angiotensin receptor blockers, diuretics, beta-blockers); (*iv*) oral contraceptive or estrogen use; (v) history of COVID-19 (including subjects with history of positive PCR test for SARS-CoV2 from nasal swab).

Eligible patients were seen between December 2020 and June 2021. After providing their informed consent to participate in this study, all the subjects underwent a medical visit and a fasting blood sample. During the medical visit the following parameters were collected: age, sex, BMI, systolic blood pressure (SBP) and diastolic blood pressure (DBP), medication, type of insulin regimen, comorbidities. Fasting blood samples were taken after a day of rest and were used to collect sera and to isolate peripheral blood mononuclear cells (PBMC). Glucose, glycated hemoglobin, cholesterol and tryglicerides were measured by autoanalyzer. RAS components were measured by real-time PCR and ELISA. This study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Institutional Review Board and Ethics Committee (CEUR-2019-SPER-113).

2.2. PBMC isolation, gene expression analysis, and ELISA

To isolate PBMC, blood samples were collected in EDTA-tubes and diluted with the same volume of Ficoll-Paque™ Plus (Cytiva Sweden AB) and then centrifuged at 2400 rpm for 30 min at room temperature. The mononuclear cell layer that was obtained was used to extract RNA.

RNA extraction was performed with the AllPrep DNA/RNA mini kit (Qiagen), according to manufacturer's instructions. A total of 700 ng of RNA were used to synthesize cDNA and quantify gene expression with RT-qPCR. The expression of ACE, ACE2 was evaluated with the TaqMan Gene Expression Assay (Life Technologies). Fluorescence for each cycle was quantitatively analyzed by StepOnePlus real-time PCR system (Applied Biosystems). Gene expression was normalized to 18s (TaqMan), and reported as a ratio compared with the level of expression in controls, which were given an arbitrary value of 1. Primer sequences are reported in the Supplementary Table.

AngII (Elabscience, E-EL-H0326) and Ang1-7 (Elabscience, E-EL-H5518), as well as circulating ACE2 (Elabscience, E-EL-H0281) were measured by ELISA, according to manufacturer's instructions.

2.3. Statistical analysis

All statistical analyses were carried out in R system for statistical computing (Ver 5.0; R development Core Team, 2018). Statistical significance was set at $p < 0.05$.

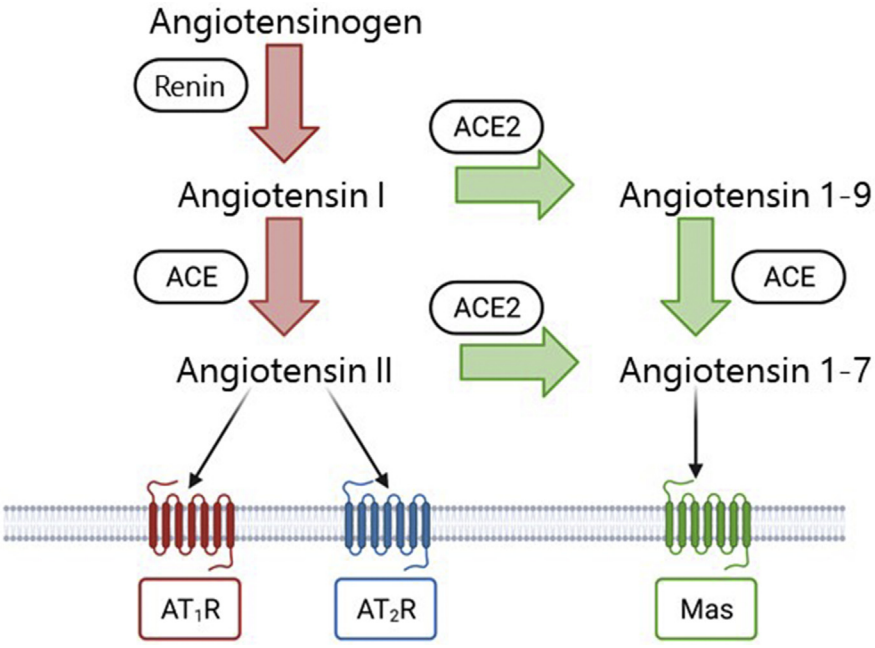

Figure 1 **Schematic representation of the key components of the renin-angiotensin system.**

Shapiro–Wilk test was applied to quantitative (continuous) variables to check for distribution normality. Continuous variables were reported as median with range (min–max) or mean ± standard deviation, depending on distribution. Categorical variables were reported as absolute frequencies and/or percentages. Continuous variables were compared by Mann–Whitney test (and Kruskall–Wallis test) or t-test (and ANOVA), depending on data distribution and number of groups. Pearson or Spearman correlation coefficients were used to evaluate linear relationships, before multivariate linear regression analyses.

3. Results

A total of 39 patients with T1D and 33 healthy controls were recruited for this study. Patients with T1D were 29-year old, had a BMI of 23.3 (17–32), and were normotensive. Controls were 32-year old, had a BMI of 22.3 (17–31) and were normotensive. Groups did not differ in terms of age, sex, BMI, SBP, and DBP. In addition, none of the women had entered menopause, and none of the study participants practiced competitive sports or underwent intensive exercise training in the weeks prior to the study enrollment, which are two conditions that may affect the expression of RAS components [15–18].

Patients with T1D had fasting glucose levels of 133 mg/dL (69–250) and their median glycated hemoglobin was 7.6% (4.9–10.8). Diabetes duration was 17 ± 8 years. They exhibited lower total and LDL cholesterol as compared to controls, according to current indications to keep LDL cholesterol <100 mg/dL. Overall, a total of 20 patients with T1D (51%) were treated with continuous subcutaneous insulin infusion (CSII), while 19 patients (49%) were treated with multiple daily injections (MDI). All the general characteristics are reported in Table 1.

Patients with T1D exhibited a significant increase of ACE and a significant decrease of ACE2 gene expression in PBMC, with a subsequent significant increase of the ACE/ACE2 ratio as compared to controls. This was associated with a significant increase of circulating AngII and the ratio AngII/Ang1-7, Fig. 2. ACE expression, as well as the ratio ACE/ACE2 and AngII levels showed a significant correlation with fasting glucose (but not cholesterol). In addition, ACE expression was also significantly related to diabetes duration (Table 2).

Table 1 Patient general characteristics.

	CNT	T1D	p-value
n	33	39	
Age (years)	32 (24–48)	29 (22–46)	0.53
Male/Female	14/19	20/19	0.64
BMI (weight/height^2)	22.3 (17–31)	23.3 (17–32)	0.42
SBP (mmHg)	115 (93–141)	115 (100–135)	0.63
DBP (mmHg)	72 (60–90)	70 (60–85)	0.21
Glucose (mg/dL)	88 (67–101)	133 (69–250)	<0.0001
HbA1c levels (%)	–	7.6 (4.9–10.8)	
Total Cholesterol (mg/dL)	200.5 (123–240)	171.5 (117–256)	0.01
HDL (mg/dL)	64 (46–92)	60.5 (34–127)	0.23
Triglycerides (mg/dL)	72.5 (40–146)	71 (39–216)	0.70
LDL (mg/dL)	120 (55–171)	96 (57–165)	0.005
ACR (mg/g)	–	3 (0–39)	

CNT is for control, T1D is for type 1 diabetes, BMI is for body mass index, SBP is for systolic blood pressure, DBP is for diastolic blood pressure, HbA1c is for glycated hemoglobin, HDL is for high density lipoprotein cholesterol, LDL is for low density lipoprotein cholesterol, ACR is for albumin creatinine ratio.

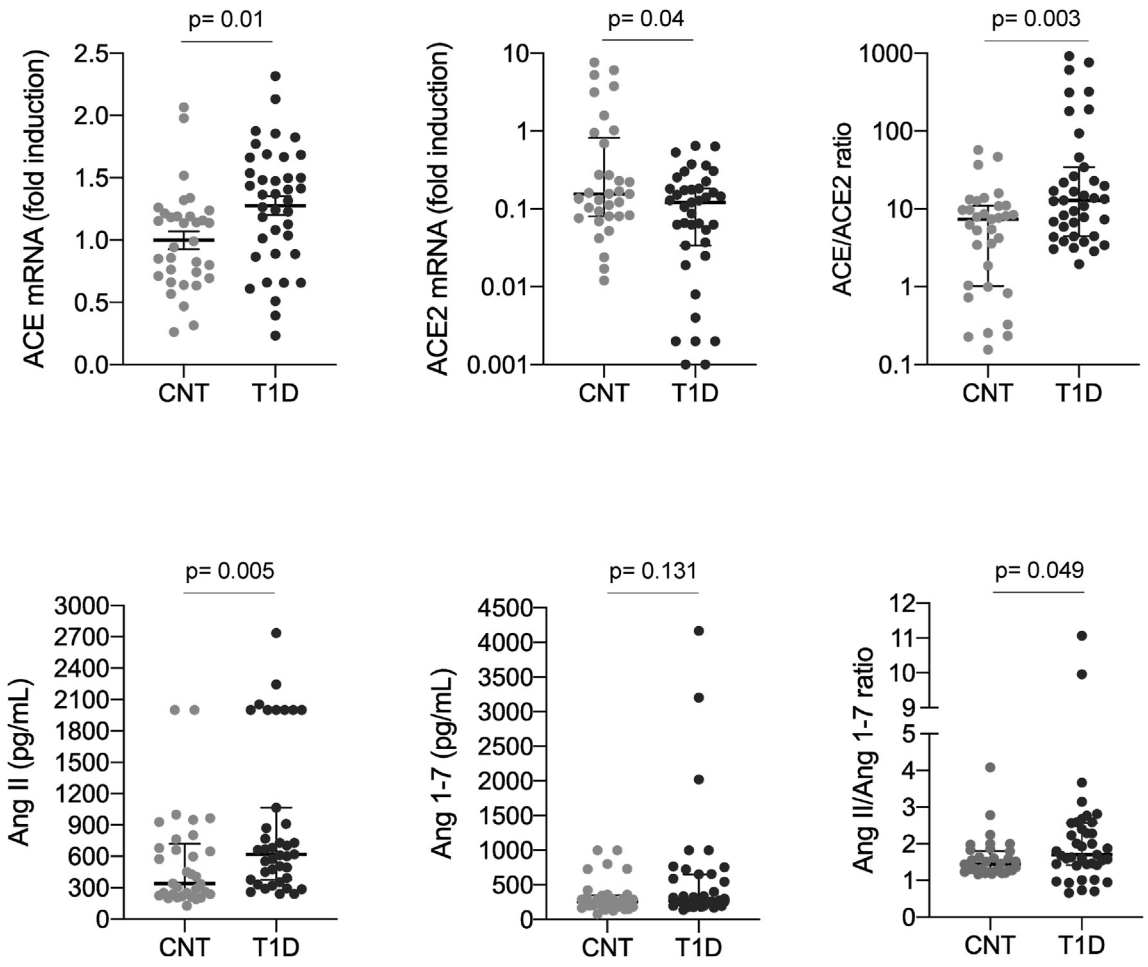

Figure 2 Gene expression of ACE, ACE2, circulating levels of AngII and Ang1-7, and their ratios in patients with T1D. Gene expression was measured in peripheral blood mononuclear cells. ACE is for angiotensin converting enzyme, Ang is for angiotensin, CNT is for controls and T1D is for type 1 diabetes.

As for sex, female participants exhibited higher levels of ACE2 gene expression and lower ACE/ACE2 ratio as compared to male subjects (Fig. 3). The linear regression confirmed the presence of an independent association between glucose and both ACE and AngII expression, while ACE2 expression was related to the sex of the participants (Table 3).

When we looked at the effect that diabetes had on the relationship between female sex and converting enzymes, we found that T1D women did not show the same increase of ACE2 expression that was seen in control women. Consistent with this, women with T1D exhibited higher ACE/ACE2 ratio as compared to control women, and the ratio ACE/ACE2 did not differ between men and women with T1D (Fig. 4).

Table 2 Correlation coefficients.

Variable	ACE		ACE2		ACE/ACE2		AngII		AngII/Ang1-7	
	rho	p	rho	p	rho	p	rho	p	rho	p
Age	−0.29	0.01	−0.01	0.93	−0.12	0.33	−0.14	0.24	−0.06	0.60
DM-Years	0.32	0.04	0.01	0.98	0.07	0.65	0.19	0.24	0.16	0.33
Glucose	0.37	0.002	−0.15	0.22	0.26	0.03	0.25	0.04	0.21	0.09
HbA1c	0.32	0.054	0.32	0.052	−0.30	0.07	0.19	0.27	0.29	0.17
TOT-cho	−0.03	0.83	0.14	0.29	−0.20	0.12	−0.11	0.42	−0.17	0.19
LDL-cho	−0.17	0.20	0.05	0.69	−0.14	0.28	−0.10	0.47	−0.15	0.25

ACE is for angiotensin-converting enzyme, Ang is for angiotensin, DM-years is for diabetes duration (years), HbA1c is for glycated hemoglobin, TOT-cho is for total cholesterol, LDL-cho is for LDL cholesterol.

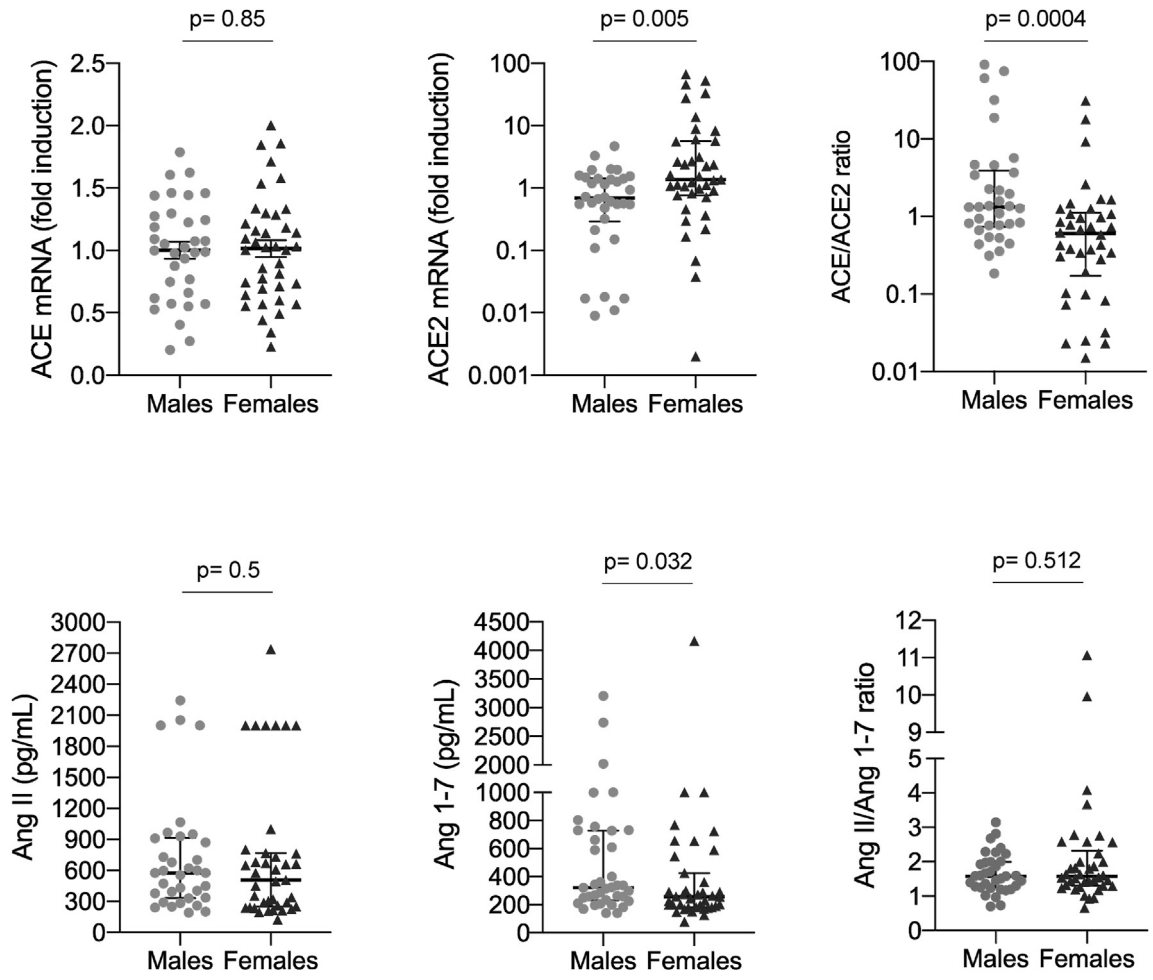

Figure 3 Sex differences of gene expression of ACE, ACE2, circulating levels of AngII and Ang1-7, and their ratios. Gene expression was measured in peripheral blood mononuclear cells. ACE is for angiotensin converting enzyme, Ang is for angiotensin.

Table 3 Linear regression model.

		Dependent variable: ACE		
Predictive variables	β-estimate	95% CI	Standard error	p-value
Age	−0.011	[-0.026, 0.003]	0.007	0.123
Sex [M]	−0.102	[-0.315, 0.112]	0.107	0.345
Glucose	0.003	[0.001, 0.006]	0.001	0.008
		Dependent variable: ACE2		
Predictive variables	β-estimate	95% CI	Standard error	p-value
Age	−0.031	[-0.075, 0.013]	0.022	0.167
Sex [M]	−0.902	[-1.553, −0.250]	0.326	0.007
Glucose	−0.006	[-0.014, 0.001]	0.004	0.097
		Dependent variable: AngII		
Predictive variables	β-estimate	95% CI	Standard error	p-value
Age	−2.920	[-24.057, 18.217]	10.587	0.783
Sex [M]	−71.236	[-384.563, 242.090]	156.933	0.651
Glucose	3.627	[0.063, 7.191]	1.785	0.046

4. Discussion

This study shows that T1D patients had higher ACE expression, circulating AngII, and AngII/Ang1−7 levels as compared to controls. Our linear regression showed that there was an independent direct association between fasting glucose levels and ACE expression or AngII levels. Our data are in line with early studies reporting an activation of the renin-angiotensin system in subjects with diabetes [19], which contributes substantially to CVD morbidity and mortality in this setting [20]. As reviewed in Ref. [11], it has been shown that hyperglycemia directly stimulates AngII production in different cell lines, such as cardiomyocytes and endothelial cells [21,22], but it also enhances the tissue responses to AngII, as it promotes the formation of advanced glycation end products which activate the angiotensin receptor AT1R [23]. In addition, in our study we found that patients with T1D had lower levels of ACE2 and higher ACE/ACE2 ratio, as compared to controls. These findings are consistent with animal studies showing that diabetes induction was associated with a

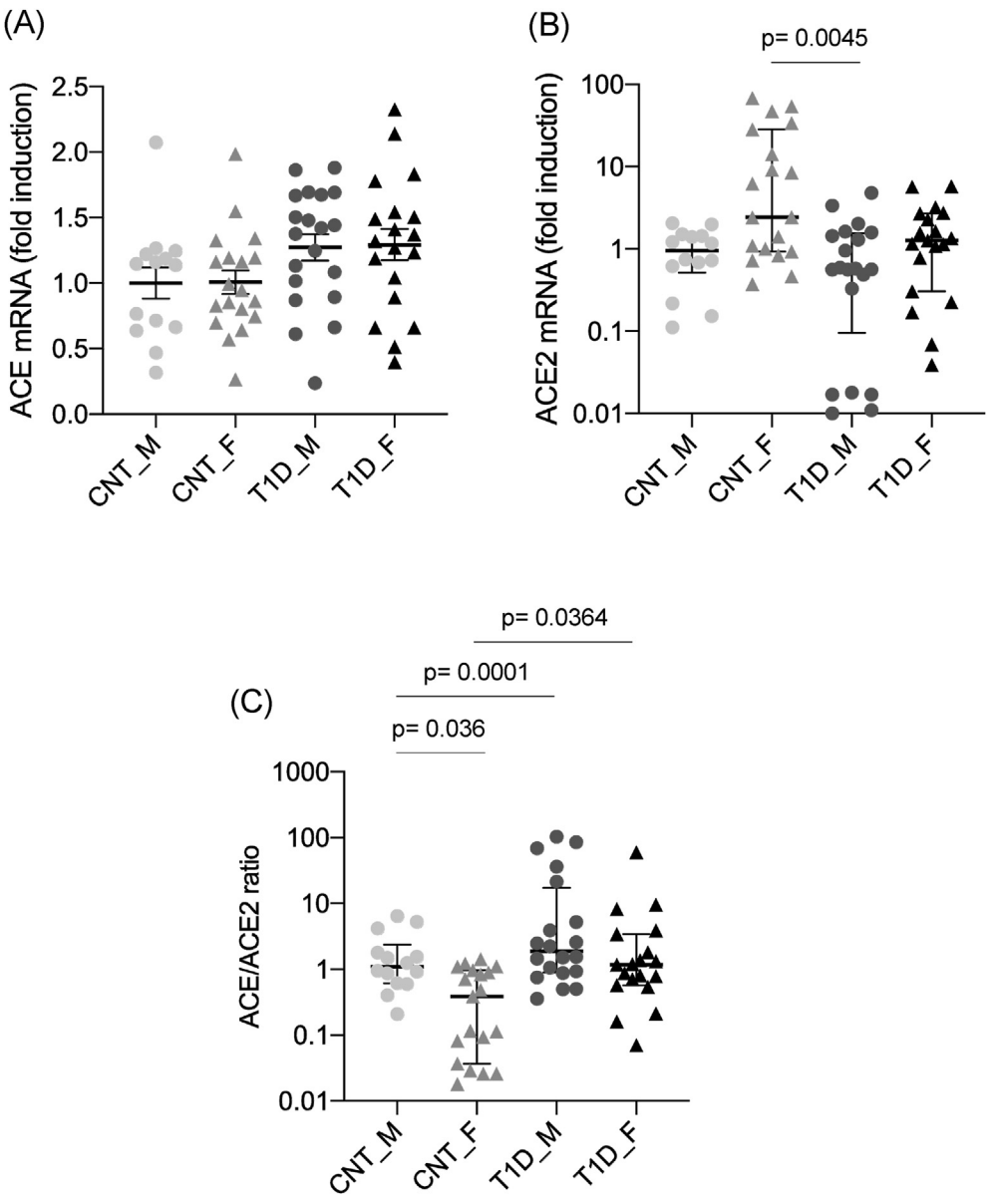

Figure 4 Impact of sex and diabetes on ACE, ACE2 and their ratio. Gene expression was measured in peripheral blood mononuclear cells. (A) One-Way ANOVA overall p-value = 0.09; (B) Kruskall–Wallis overall p-value = 0.0045; (C) Kruskall–Wallis overall p-value = 0.0002.

reduction of renal and cardiac ACE2 expression, leading to a decrease of circulating Ang1–7 and to an elevation of AngII [14,24,25]. Interestingly, the effect that T1D had on ACE2 can be considered a third mechanism whereby diabetes promotes AngII actions, as ACE2 downregulation impairs AngII clearance, with subsequent increase of ACE/ACE2 and AngII/Ang1-7 ratios.

Interestingly, our study shows that ACE2 expression was significantly related also to the sex of participants, being significantly higher in the female group, and independently associated with the sex of participants at the linear regression analysis. This is due to the fact that ACE2 gene is located on the X chromosome, and the X chromosome inactivation that should silence the transcription from one of the two X chromosomes in female mammalian cells, is often incomplete [26]. It has been shown that incomplete X chromosome inactivation affects at least 23% of X-chromosomal genes, resulting in sex-biases in gene expression underlying sex-related phenotypic diversity [26]. The higher ACE2 expression that is observed in women could justify the gender susceptibility to COVID-19, and the higher severity and mortality of COVID-19 that was seen in males as compared to females [27]. It has been argued that both SARS-CoV and SARS-CoV2 bind to ACE2 and enter the cells via endocytosis [7,28], such that the initial detrimental effects of viral infection begins with a loss of ACE2-mediated tissue protection against proinflammatory Ang II. Therefore, higher expression of ACE2 leading to lower ACE/ACE2 ratio can actually protect against the development of severe tissue injury and severe forms of COVID-19, once the subject gets the infection [28].

Our data indicate that not only sex but also T1D was significantly associated with ACE2 expression. When we looked at the effect that diabetes had on ACE2 expression in the male and female groups, we found that ACE2 expression failed to increase in women with T1D as compared to control women. Consistent with this, women with T1D showed significantly higher ACE/ACE2 levels as compared to control women, and no differences as compared to men with T1D. This might be related to the fact that even transient high glucose causes persistent epigenetic changes and altered gene expression [29], interfering with incomplete X chromosome inactivation. Nevertheless, the fact that ACE2 gene expression failed to increase in females with T1D, as compared to their controls, with subsequent increase of ACE/ACE2 ratio, which did not differ between men and women with T1D, might explain why CVD or COVID-19 do not show substantial gender differences in patients with diabetes.

The limitations of this study include the fact that ACE and ACE2 expression was measured in PBMC, and not in other tissues, such as the lungs, heart and vessels. This was due to the fact that PBMC represent the most easily accessible tissue in patients. Another issue is the well-known variability of the measured parameters, such as angiotensins, which are produced by several organs, and whose levels are influenced by several factors, such as physical exercise [15,16] as well and estradiol levels [17,18]. Nevertheless, this is the first study evaluating ACE2 in patients with type 1 diabetes, whose strengths include the selection of young T1D patients, with median HbA1c of 7.6%, and no significant comorbidities. In addition, none of the women had entered menopause and none of the subjects practiced competitive sport or had history of intense exercise training prior to the study enrollment.

In conclusion, our data indicate that T1D significantly affects the renin-angiotensin system, promoting the expression of ACE and AngII actions, which might contribute to the higher cardiovascular risk of T1D patients, as well as to severe tissue injury induced by SARS-CoV2. Our data might also explain why CVD or COVID-19 do not show substantial gender differences in patients with T1D given that ACE2 gene expression failed to increase in females with T1D, with subsequent increase of ACE/ACE2 ratio and no differences between men and women with diabetes.

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Declaration of competing interest

The Authors have no competing interest or conflict of interest to declare.

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