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TRABALHOS RELEVANTES

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Trabalhos mais relevantes

MANUELA SOFIA RODRIGUES MORATO

PROVAS DE AGREGAÇÃO
FACULDADE DE FARMÁCIA DA UNIVERSIDADE DO PORTO

Trabalhos mencionados no currículo
considerados pelo candidato como os mais relevantes,
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atribuição do título académico de Agregado em Ciências Farmacêuticas,
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Índice

TRABALHO 1

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Angiotensin II contributes to glomerular hyperfiltration in diabetic rats independently of adenosine type I receptors

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Patinha D, Fasching A, Pinho D, Albino-Teixeira A, Morato M, Palm F. Angiotensin II contributes to glomerular hyperfiltration in diabetic rats independently of adenosine type I receptors. *Am J Physiol Renal Physiol* 304: F614–F622, 2013. First published January 2, 2013; doi:10.1152/ajprenal.00285.2012.—Increased angiotensin II (ANG II) or adenosine can potentiate each other in the regulation of renal hemodynamics and tubular function. Diabetes is characterized by hyperfiltration, yet the roles of ANG II and adenosine receptors for controlling baseline renal blood flow (RBF) or tubular Na^+ handling in diabetes is presently unknown. Accordingly, the changes in their functions were investigated in control and 2-wk streptozotocin-diabetic rats after intrarenal infusion of the ANG II AT_1 receptor antagonist candesartan, the adenosine A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), or their combination. Compared with controls, the baseline blood pressure, RBF, and renal vascular resistance (RVR) were similar in diabetics, whereas the glomerular filtration rate (GFR) and filtration fraction (FF) were increased. Candesartan, DPCPX, or the combination increased RBF and decreased RVR similarly in all groups. In controls, the GFR was increased by DPCPX, but in diabetics, it was decreased by candesartan. The FF was decreased by candesartan and DPCPX, independently. DPCPX caused the most pronounced increase in fractional Na^+ excretion in both controls and diabetics, whereas candesartan or the combination only affected fractional Li^+ excretion in diabetics. These results suggest that RBF, via a unifying mechanism, and tubular function are under strict tonic control of both ANG II and adenosine in both control and diabetic kidneys. Furthermore, increased vascular AT_1 receptor activity is a contribution to diabetes-induced hyperfiltration independent of any effect of adenosine A_1 receptors.

AT_1 receptors; A_1 receptors; tubular function; Na^+ handling; diabetes

ADENOSINE AND THE RENIN-ANGIOTENSIN system (RAS) are involved in the regulation of intrarenal hemodynamics and tubular function (18, 53). It is well-known that activation of either angiotensin II (ANG II) AT_1 receptors or adenosine A_1 receptors alters kidney function (18, 53). ANG II, acting on AT_1 receptors, reduces glomerular filtration rate (GFR) and renal blood flow (RBF) by increasing pre- and postglomerular resistances (8, 44). Indeed, the most commonly used treatment to reduce the progression of proteinuric kidney disease in diabetics includes targeting the RAS (3). Adenosine controls renin release (2, 15, 25, 48), has direct vascular effects (1, 32, 33, 38), and is a fundamental component of the tubuloglomerular feedback (TGF) mechanism (46, 53).

Diabetic nephropathy is a leading cause of morbidity and mortality. Since the initial glomerular hyperfiltration (29) is crucial for the progression of renal injury (24), it is very important to establish and target its cause yet the mechanism remains unclear. Reduced preglomerular vascular resistance has been emphasized (7) and commonly attributed to the combination of a reduced TGF (52) and myogenic response of the afferent arteriole (14). Adenosine, through activation of adenosine A_1 receptors, is the main effector of the TGF-induced afferent arteriolar vasoconstriction (46, 54). However, diabetic adenosine A_1 receptor knockout mice lack the TGF mechanism and still develop hyperfiltration (45), suggesting that TGF is not the only mechanism involved (39). Also, in early diabetic nephropathy there is increased proximal Na^+ reabsorption (13) associated with enhanced expression of adenosine A_1 receptors (37), tubular hypertrophy, and increased Na^+/glucose cotransport (52), which may also contribute to hyperfiltration (39) due to the decreased pressure in the Bowman's space that enhances the net ultrafiltration pressure by a nonvascular mechanism.

Several studies have addressed the roles of ANG II and adenosine, and their interaction, in controlling vascular tone using either isolated microvessels, in situ perfused kidneys, pathological alterations or systemic manipulation of these vasoactive systems (12, 36, 49, 56). However, the in vivo significance of tonic baseline activation of ANG II and adenosine receptors, and any interaction between the two, are presently unknown, partly due to the systemic effects of blocking these receptors. Furthermore, its relevance for the functional alterations occurring in the diabetic kidney has not previously been demonstrated. Of importance also are the effects of both ANG II and adenosine to enhanced proximal tubule reabsorption via the Na^+/H^+ exchanger NHE3. These might act in parallel with hyperglycemia-induced Na^+-glucose reabsorption to provide a nonvascular site for interaction between the two systems that can only be studied in the intact kidneys of diabetic models. Therefore, the aim of the present study was to elucidate the role of in vivo tonic activation of ANG II and adenosine receptors on renal hemodynamics and tubular function both in control and diabetic rats. For that reason, we developed a protocol (Fig. 1*B*) in which the drugs were infused directly into the renal artery in a slow step-wise manner, allowing to directly investigate the effects on intrarenal hemodynamics and tubular function without confounding

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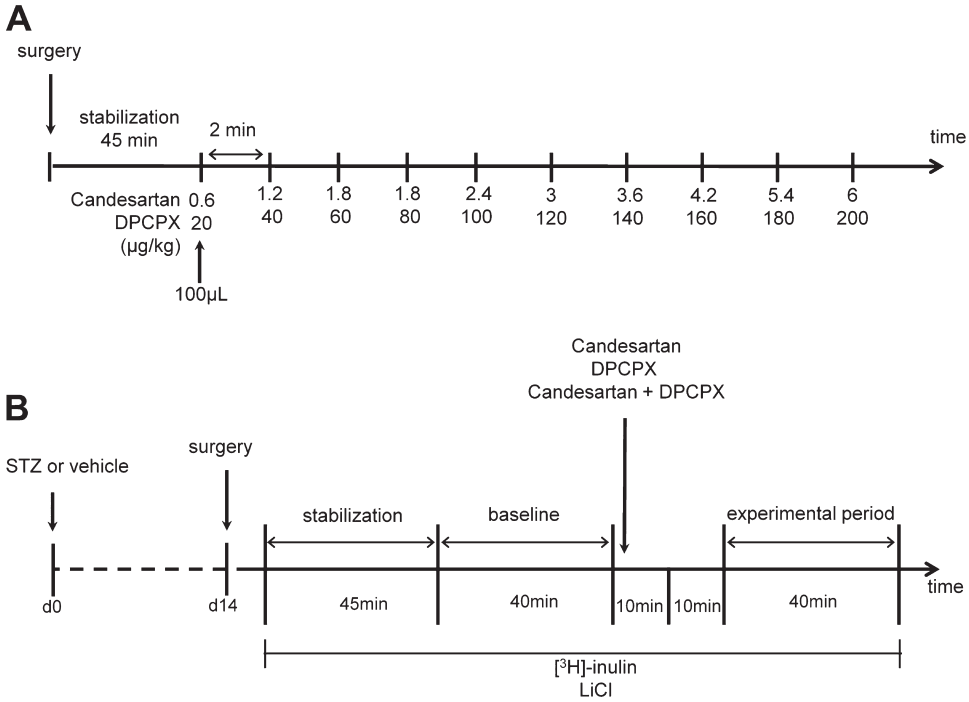

Fig. 1. Outline of the two protocols. *A*: protocol used for determining the dose-response curve for the angiotensin II (ANG II) AT_1 receptor antagonist candesartan, or the adenosine A_1 receptor antagonist 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX), or the combination of the two. *B*: protocol used for investigating the roles of AT_1 and A_1 receptors on the regulation of intrarenal hemodynamics and tubular Na^+ handling. STZ, streptozotocin.

systemic effects. This is important since even small changes in arterial pressure can affect proximal reabsorption and renal hemodynamics.

MATERIALS AND METHODS

All chemicals were from Sigma Aldrich (St. Louis, MO) and of highest grade available if not otherwise stated.

Animal model. Male Sprague-Dawley rats (300–350 g; Charles River) with free access to water and standard rat chow were injected with streptozotocin (50 mg/kg) or vehicle into the tail vein. Blood glucose concentration was measured using test reagent strips (MediSense, Bedford, MA). Animals were considered diabetic if blood glucose concentration was and remained >18 mmol/l within 48 h after streptozotocin injection. Experiments were conducted 14 days after induction of diabetes. All experiments were performed in accordance with the National Institutes of Health *Guidelines for Use and Care of Laboratory Animals* and approved by the local Animal Care and Use Committee.

Dose-response curve for candesartan and 8-cyclopentyl-1,3-dipropylxanthine. Normoglycemic animals were anesthetized with thiobutabarbital (120 mg/kg ip; Inactin) and surgically prepared as previously described (28). Briefly, animals were placed on a heated operating table at 38°C and tracheostomized. Polyethylene catheters were placed in the left femoral vein for infusion of Ringer solution (5 ml·kg body $wt^{-1}\cdot h^{-1}$ for controls and 10 ml·kg body $wt^{-1}\cdot h^{-1}$ for diabetics) and in the left femoral artery for blood pressure measurements. The bladder was catheterized for free urinary drainage of the right kidney. The left kidney was exposed by a left subcostal flank incision, immobilized in a plastic cup, and embedded in pieces of saline-soaked cotton wool, and the surface was covered with paraffin oil (Apoteksbolaget, Gothenburg, Sweden) to prevent evaporation and keep the tissue moist at body temperature. Thereafter, a catheter was advanced ~1–2 mm into the left renal artery, through a lumbar artery, for kidney-specific delivery of drugs. All infusion solutions administered into the renal artery contained lissamine green to visually verify homogenous intrarenal distribution of the vasoactive substances. The ANG II AT_1 receptor antagonist candesartan (AstraZeneca, Mölndal, Sweden) was administered in doses between 0.6 and 6.0 μg/kg and the adenosine A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) at doses between 20 and 200 μg/kg to separate animals. DPCPX was dissolved in saline with a final concentration of 1% DMSO. A third group of animals received a combination of both antagonists. After 45 min of stabilization, each dose was infused every 2 min in a total volume of 100 μl (Fig. 1*A*).

In vivo intrarenal blockade of ANG II and adenosine receptors. Control and diabetic animals were anesthetized with thiobutabarbital (Inactin; 120 and 80 mg/kg ip for normoglycemic and hyperglycemic animals, respectively) and surgically prepared as described above. [^{3}H]inulin (185 $kBq\cdot kg^{-1}\cdot h^{-1}$; ARC, St. Louis, MO) and LiCl (4 mg ip bolus plus continuous infusion 2.1 $mg\cdot h^{-1}\cdot rat^{-1}$; Ref. 28) were infused in the femoral vein. After 45 min of stabilization, a 40-min period for baseline measurements was followed by an additional 40-min experimental period in which candesartan (4.2 μg/kg), DPCPX (140 μg/kg), or both were infused in the renal artery in a total volume of 700 μl. Since the half-life of candesartan is approximately 9 h (6) and that of DPCPX ~5 h (17), a single bolus dose of each drug was infused and full receptor blockade assumed for the duration of the experiments. To minimize spill-over to the systemic circulation, the infusion was stepwise during ~10 min followed by a 10-min delay before the experimental period started (Fig. 1*B*). Blood samples for measurement of blood gas parameters were collected at the end of each period.

In some animals, the experimental period of candesartan or DPCPX was followed by a second experimental period in which the ANG II AT_2 receptor antagonist *S*-(+)-1-{[4-(dimethylamino)-3-methylphenyl]methyl}-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid di(trifluoroacetate) salt hydrate (PD-123,319; 0.5 mg/kg) or the adenosine A_2 receptor antagonist 3,7-dimethyl-1-propargylxanthine (DMPX; 0.5 mg/kg) was administrated, respectively. In these experiments, the blood gas parameters were not assessed due to the large volume of blood required for these measurements.

Measurements of kidney hemodynamics and tubular function. Mean arterial blood pressure (MAP) was measured using a transducer (model P23dB; Statham Laboratories, Los Angeles, CA) connected to the left femoral artery catheter. Total RBF was measured using an ultrasound probe (Transonic Systems, Ithaca, NY) placed around the

Table 1. *Comparison between control and diabetic rats during baseline*

	Control Rats	Diabetic Rats
Blood glucose, mM	6.5 ± 0.3	23.7 ± 0.5*
Body weight, g	377 ± 5	355 ± 4*
Left kidney weight, g	1.24 ± 0.02	1.73 ± 0.03*
MAP, mmHg	108 ± 2	107 ± 2
RBF, ml/min	8.4 ± 0.2	8.4 ± 0.2
RVR, mmHg·min^{-}·ml	13.2 ± 0.4	13.2 ± 0.4
GFR, ml/min	1.6 ± 0.1	3.5 ± 0.2*
FF, %	10.9 ± 0.9	21.9 ± 1.2*
Urine flow, μl/min	5.4 ± 0.6	28.6 ± 1.8*
T_{Na}, μmol/min	226 ± 17	492 ± 27*
FE_{Na}, %	0.2 ± 0.0	0.3 ± 0.0*
FE_{Li}, %	40.9 ± 5.6	24.5 ± 3.0*
$Q\{\downarrow O_2\}$, μmol/min	8.8 ± 1.1	14.0 ± 1.4*

Results are expressed as means ± SE for $n = 34–44$, except for oxygen consumption ($Q\{\downarrow O_2\}$) where $n = 22–23$. MAP, mean arterial pressure; RBF; renal blood flow; RVR, renal vascular resistance; GFR, glomerular filtration rate; FF, filtration fraction; T_{Na}, tubular Na^+ transport; FE_{Na}, fractional Na^+ excretion; FE_{Li}, fractional Li^+ excretion. *$P < 0.05$.

left renal artery; care was taken so that the probe remained in the same position throughout the protocol. These parameters were continuously recorded with a Power Lab instrument (AD Instruments, Hastings, UK). GFR was determined by [^{3}H]inulin clearance. ^{3}H activities in urine and plasma were measured by liquid scintillation. Blood gas parameters were measured from samples drawn from the right femoral artery and left renal vein using an iSTAT System (Abbott Laboratories; Ref. 35). The left vein blood sample was carefully collected using an angled pointed needle connected to a preheparinized syringe. Tubular Na^+ reabsorption was determined using Li^+ clearance (50). Kidney weights were determined at the end of the experiment. Urine volumes were measured gravimetrically, and urinary Na^+ and Li^+ and plasma Li^+ concentrations were quantified by flame spectrophotometry in a multianalyzer (model IL543; Instrumentation Lab, Milan, Italy).

Calculations. GFR was calculated using the formula GFR = U * V/P, where U and P denote the quantity of [^{3}H]inulin in the urine and plasma samples, respectively, and V denotes the urine flow rate. Renal vascular resistance (RVR) was calculated as MAP divided by total RBF. The filtration fraction (FF) was calculated using the formula FF = GFR/[total RBF * (1 − Hct)]. In vivo renal oxygen consumption was estimated from the arteriovenous difference in O_2 content {O_{2ct} = ([Hb] * oxygen saturation * 1.34 + * Po_2 0.003) * total RBF}. Tubular Na^+ transport (T_{Na}) was calculated as $T_{Na} = [P_{Na}] * GFR - [U_{Na}] *$ urine flow, where $[P_{Na}]$ and $[U_{Na}]$ are plasma and urine Na^+ concentration, respectively. Fractional Na^+ excretion (FE_{Na}) was estimated from $[U_{Na}] * [P_{inulin}]/[P_{Na}] * [U_{inulin}]$, and fractional Li^+ excretion (FE_{Li}) from $[U_{Li}] * [P_{inulin}]/[P_{Li}] * [U_{inulin}]$, where $[P_{inulin}]$ and $[U_{inulin}]$ represent plasma and urinary inulin concentration, and $[P_{Li}]$ is plasma Li^+ concentration.

Statistical evaluation. Multiple comparisons within the same group were performed using one-way ANOVA for repeated measurements followed by Dunnett's post hoc test in the dose-response curves. Differences between the groups during baseline or in response to a treatment were evaluated by two-way ANOVA followed by Tukey's test. Comparison within the same group before and after the treatment was performed using Student's paired *t*-test or one-way ANOVA for repeated measurements followed by Tukey's test, as appropriated. Data presented in Table 1 were analyzed using unpaired Student's *t*-test. Descriptive statistics are presented as means ± SE. For all comparisons, $P < 0.05$ was considered statistically significant.

RESULTS

Dose-response curves for the AT_1 receptor antagonist candesartan, A_1 receptor antagonist DPCPX or the combination of the two antagonists. Intrarenal infusions of Candesartan, DPCPX and their combination dose-dependently increased RBF and decreased RVR without affecting MAP (Fig. 2). Maximal effects were obtained with 4.2 μg/kg of candesartan and 140 μg/kg of DPCPX, which were therefore chosen for the subsequent experiments.

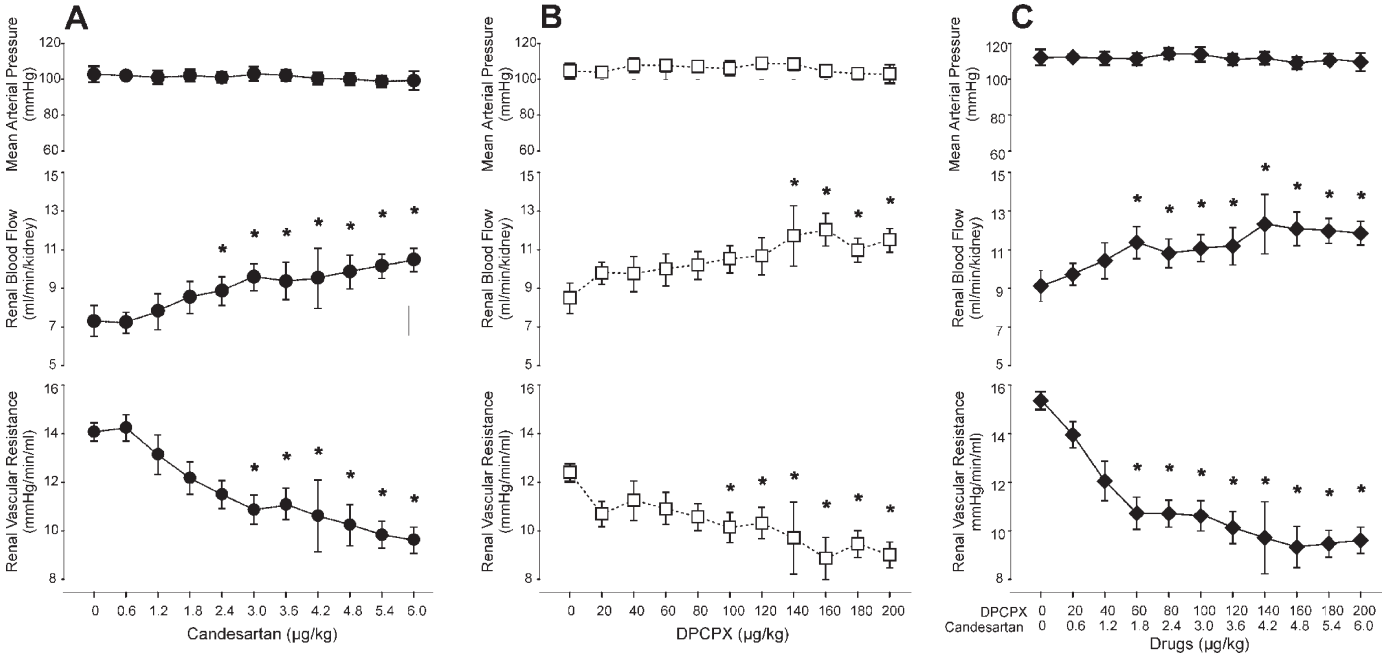

Fig. 2. Dose-response curves for the AT_1 receptor antagonist candesartan (*A*), the A_1 receptor antagonist DPCPX (*B*), or the combination of the two (*C*) on mean arterial pressure, total renal blood flow and renal vascular resistance. Results expressed as means ± SE for $n = 5–9$/group. *$P < 0.05$ vs. *time 0*.

In vivo intrarenal blockade of ANG II and adenosine receptors: vascular effects. Streptozotocin increased blood glucose, decreased body weight, and increased kidney weight compared with controls (Table 1). Remarkably, although baseline MAP, RBF, and RVR were similar, the GFR of diabetic rats was doubled compared with the controls, resulting in a doubling of the filtration fraction (FF) (Table 1). Candesartan reduced MAP in control (~4 mmHg) and diabetic (~5 mmHg) animals, as did the combination of the two antagonists in controls (~6 mmHg; Fig. 3). DPCPX had no effect on MAP in either group (Fig. 3). Candesartan, DPCPX, or the combination increased RBF by ~33%,

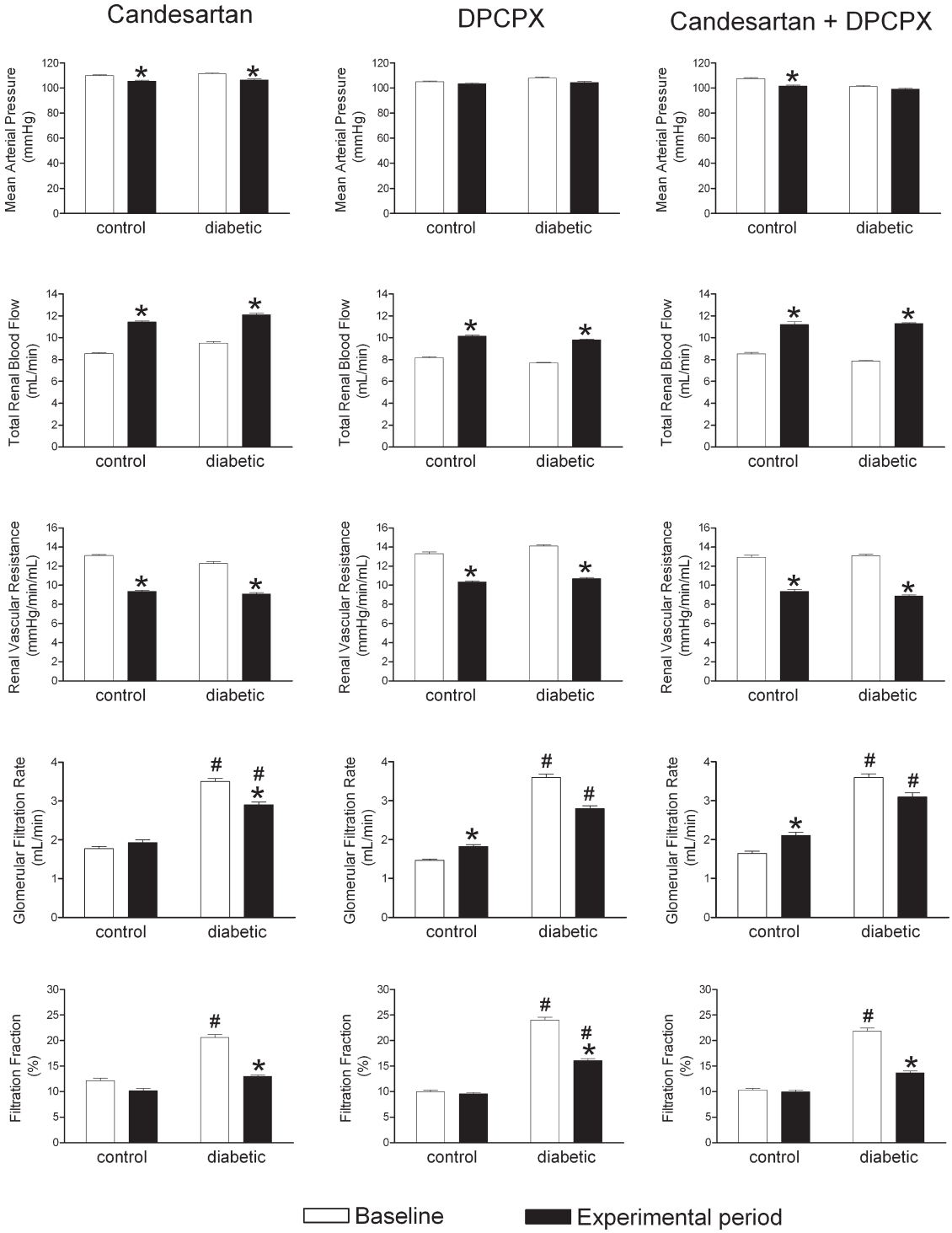

Fig. 3. Vascular effects of the in vivo intrarenal blockade of ANG II and adenosine receptors. Mean arterial pressure, total renal blood flow, renal vascular resistance, glomerular filtration rate, and filtration fraction of control and diabetic animals before and after blockade of AT_1 receptors by candesartan (4.2 μg/kg), A_1 receptors by DPCPX (140 μg/kg), or the combined blockade of AT_1 and A_1 receptors. Results expressed as means ± SE for $n = 8$–16. *$P < 0.05$ vs. baseline within the same group. #$P < 0.05$ vs. corresponding control.

Table 2. *Renal effects of the intrarenal blockade of ANG II AT_1 and AT_2 receptors in control and diabetic rats*

	Control Rats			Diabetic Rats		
	Baseline	Candesartan	Candesartan + PD-123,319	Baseline	Candesartan	Candesartan + PD-123,319
MAP, mmHg	120 ± 4	116 ± 5	111 ± 5*†	114 ± 4	111 ± 3	108 ± 3*
RBF, ml/min	8.8 ± 0.6	11.6 ± 0.4*	11.0 ± 0.4*	9.9 ± 0.8	12.7 ± 1.0*	12.2 ± 1.0*
RVR, mmHg·min^{-}·ml	13.8 ± 0.8	10.0 ± 0.4*	10.1 ± 0.4*	12.1 ± 1.2	9.3 ± 1.0*	9.3 ± 0.9*
GFR, ml/min	2.3 ± 0.3	2.2 ± 0.3	2.1 ± 0.3	3.5 ± 0.2‡	3.3 ± 0.4‡	2.7 ± 0.3
FF, %	40 ± 4	32 ± 3	36 ± 7	69 ± 8‡	46 ± 4*	41 ± 7*
Urine flow, μl/min	11 ± 2	15 ± 2*	11 ± 2†	27 ± 5‡	21 ± 3	22 ± 3‡
T_{Na}, %	320 ± 47	302 ± 35	295 ± 35	488 ± 35‡	486 ± 60‡	381 ± 50
FE_{Na}, %	0.5 ± 0.1	0.8 ± 0.2*	0.6 ± 0.1*	0.6 ± 0.1	0.9 ± 0.2	1.1 ± 0.2

Results are expressed as means ± SE for $n = 5$–7. Effects during baseline and after blockade of AT_2 receptors with PD-123,319 (0.5 mg/kg) superimposed on prior AT_1 receptor blockade by candesartan (4.2 μg/kg). *$P < 0.05$ vs. baseline within the same group. †$P < 0.05$ vs. candesartan within the same group. ‡$P < 0.05$ vs. corresponding control.

resulting in a reduction of RVR by ~27% in both controls and diabetics (Fig. 3).

In controls, DPCPX and combination of the two antagonists, but not candesartan alone, increased GFR, whereas FF remained unaffected (Fig. 3). In diabetics, candesartan, but neither DPCPX nor the combination, reduced GFR (Fig. 3). Although the absolute decrease in GFR was similar when inhibiting AT_1 and A_1 receptors in diabetic rats (−0.71 ± 0.29 ml/min; $n = 14$; $P = 0.028$ vs. −0.79 ± 0.40 ml/min; $n = 13$; $P = 0.073$), only candesartan resulted in a statistical significant decrease due to the more heterogeneous response in the DPCPX group. Furthermore, also the combination of candesartan and DPCPX failed to statistically reduce GFR (−0.53 ± 0.37 ml/min; $n = 11$; $P = 0.178$) due to a large variability in the response. However, FF was reduced by all three treatments administered to diabetics but only normalized following candesartan or the combination of candesartan and DPCPX (Fig. 3).

Superimposed infusion of PD-123,319 to that of candesartan or DMPX to that of DPCPX caused no relevant alterations to the effects observed with candesartan or DPCPX alone (Table 2 and 3).

In vivo intrarenal blockade of ANG II and adenosine receptors: tubular effects. Diabetics had higher baseline urine flow and FE_{Na} doubled the rate of net tubular Na^+ transport. This remarkable increase in T_{Na} must reflect increased Na^+ filtration and enhanced tubular Na^+ reabsorption. This can be ascribed predominantly to the proximal tubule since the clearance of lithium (primarily regulated by proximal reabsorption) was reduced significantly in the diabetic rats (Table 1). All three treatments increased urine flow and FE_{Na} in controls (Fig. 4). In diabetics, candesartan decreased urine flow and T_{Na}, whereas DPCPX and the combination of the two antagonists had no effect, except for increasing FE_{Na} (Fig. 4). Baseline FE_{Li} was lower and Q_{O_2} higher in diabetics compared with controls (Table 1), indicating higher proximal tubule reabsorption in diabetics. Candesartan and the combination increased FE_{Li} in diabetics (Fig. 4), indicating decreased proximal tubule reabsorption. This was associated with decreased GFR and FF. Neither candesartan nor DPCPX alters oxygen consumption, but the combination of the two increased Q_{O_2} in both controls and diabetics (Fig. 4).

Superimposed treatment with PD-123,319 to that of candesartan normalized urine flow in controls (Table 2). Superimposed treatment with DMPX to that of DPCPX normalized urine flow both in control and diabetic animals and attenuated the increase in FE_{Na} in controls (Table 3). T_{Na} was not altered by any treatment either in controls or in diabetics (Tables 2 and 3).

DISCUSSION

The main novel finding of the present study is that ANG II, acting on AT_1 receptors, and adenosine, acting on A_1 receptors, strongly influence renal hemodynamics during baseline conditions in both control and diabetic kidneys via a unifying pathway that requires both receptors to affect vascular tone. The present study also suggests increased AT_1 receptor signaling as a crucial mechanism for the elevated FF and glomerular hyperfiltration commonly observed in the diabetic kidney. To the best of our knowledge, this is the first time that the interaction between ANG II AT_1 and adenosine A_1 receptors in regulating intrarenal hemodynamics is demonstrated in vivo in diabetic

Table 3. *Renal effects of the intrarenal blockade of adenosine A_1 and A_2 receptors in control and diabetic rats*

	Control Rats			Diabetic Rats		
	Baseline	DPCPX	DPCPX + DMPX	Baseline	DPCPX	DPCPX + DMPX
MAP, mmHg	110 ± 4	111 ± 3	109 ± 3	116 ± 3	111 ± 4*	111 ± 4*
RBF, ml/min	7.4 ± 0.3	9.7 ± 0.3*	9.6 ± 0.3*	7.8 ± 0.2	10.1.±0.2*	9.8 ± 0.3*
RVR, mmHg·min^{-}·ml	14.9 ± 0.7	11.4 ± 0.5*	11.5 ± 0.4*	14.9 ± 0.5	11.0 ± 0.4*	11.4 ± 0.5*
GFR, ml/min	1.5 ± 0.2	2.0 ± 0.3	1.7 ± 0.2	3.7 ± 0.4‡	2.9 ± 0.2‡	2.5 ± 0.4‡
FF, %	38 ± 5	37 ± 4	33 ± 3	72 ± 9‡	53 ± 5*	54 ± 10‡
Urine flow, μl/min	5 ± 1	16 ± 2*	8 ± 1†	24 ± 4‡	38 ± 7*‡	28 ± 4†‡
T_{Na}, %	200 ± 26	264 ± 37	228 ± 31	507 ± 59‡	396 ± 31‡	391 ± 61‡
FE_{Na}, %	0.1 ± 0.0	1.3 ± 0.2*	0.7 ± 0.1*†	0.3 ± 0.1‡	1.4 ± 0.3*	1.3 ± 0.3*‡

Results are expressed as means ± SE for $n = 6$–8. Effects during baseline and after blockade of A_2 receptors by 3,7-dimethyl-1-propargylxanthine (DMPX; 0.5 mg/kg) superimposed on prior A_1 receptor blockade by 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 140 μg/kg). *$P < 0.05$ vs. baseline within the same group. †$P < 0.05$ vs. DPCPX within the same group. ‡$P < 0.05$ vs. corresponding control.

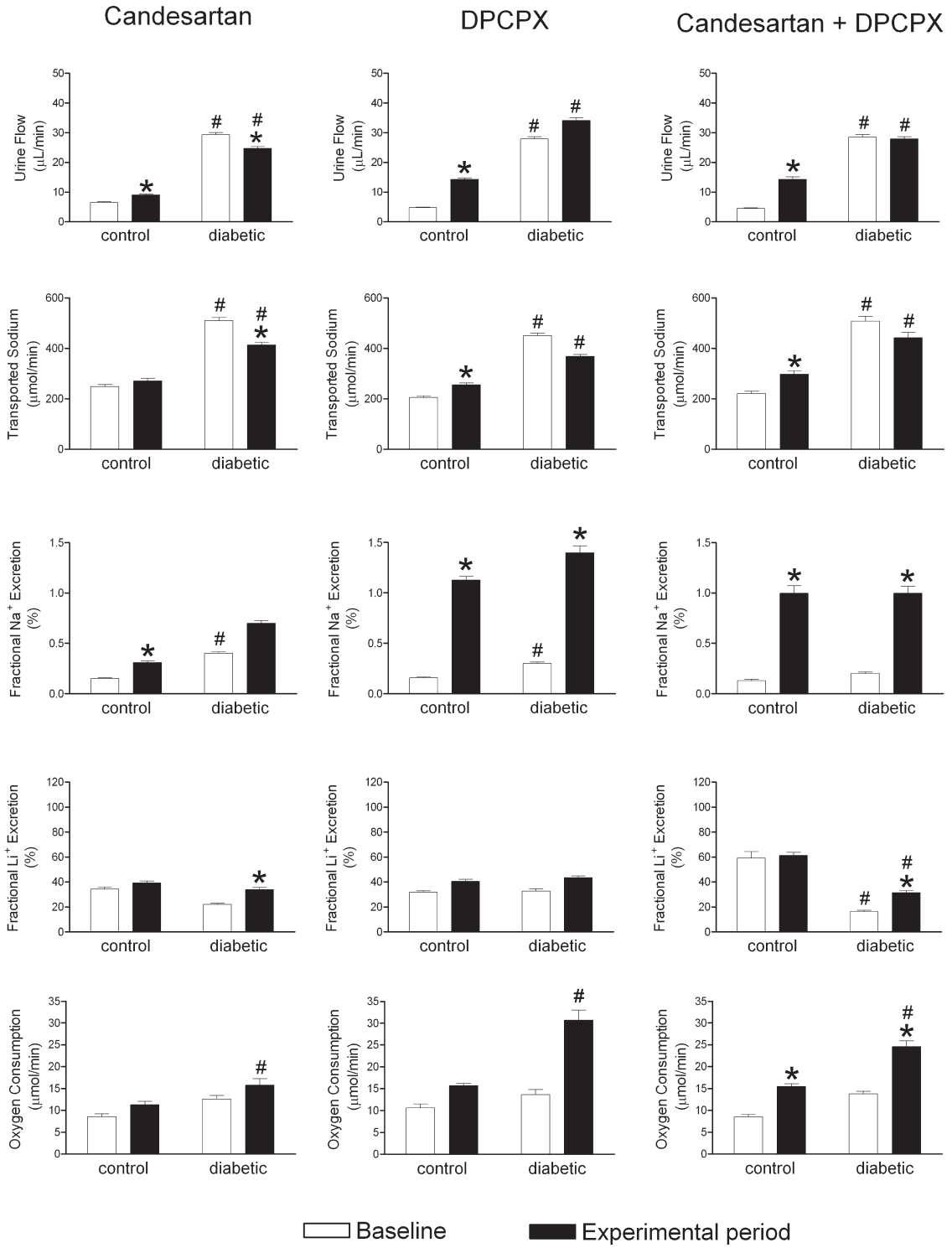

Fig. 4. Tubular effects of the in vivo intrarenal blockade of ANG II and adenosine receptors. Urine flow, fractional urinary Na^+ excretion, fractional urinary Li^+ excretion, transported Na^+ and total kidney oxygen consumption of control and diabetic animals before and after blockade of AT_1 receptors by candesartan (4.2 μg/kg), A_1 receptors by DPCPX (140 μg/kg), or the combined blockade of AT_1 and A_1 receptors. Results expressed as means ± SE for $n = 8$–16. *$P < 0.05$ vs. baseline within the same group. #$P < 0.05$ vs. corresponding control.

animals. Moreover, these data also propose that endogenous ANG II and adenosine have different roles in regulating tubular sodium handling in diabetics compared with controls.

Concomitant blockade of ANG II AT_1 and adenosine A_1 receptors caused a similar effect to the observed when only adenosine A_1 receptors were blocked. Several studies have addressed and demonstrated an interaction between ANG II AT_1 and adenosine A_1 receptors in the regulation of renal vascular tone (26, 36, 56). Indeed, inhibition of ANG II formation or ANG II AT_1 receptor signaling reduces adenosine

A_1 receptor-mediated vasoconstriction of the renal vasculature (26, 56). Conversely, ANG II AT_1 receptor-mediated vasoconstriction is also reduced by adenosine A_1 receptor blockade (21). Previous studies have been performed ex vivo or in vivo with systemic administration of the vasoactive substances. Other studies evaluated the responses to increasing doses of exogenous ANG II or adenosine, which may activate other receptor subtypes, counteracting vasoactive mechanisms (22, 27), or nonreceptor-dependent mechanisms (20). However, the present study is, to the best of our knowledge, the first report demonstrating a tonic baseline interaction between these two systems without having any confounding systemic effects or manipulation to increase receptor signaling. Importantly, concomitant blockade of both ANG II AT_1 and adenosine A_1 receptors resulted in increased RBF, which was of the same magnitude as with either drug alone. These results add further support to a unifying vasodilatory pathway in which both receptor types need to be stimulated to affect vascular tonus.

Unaltered GFR in conjunction with decreased FF suggests that these two systems regulate both afferent and efferent arterioles. However, blockade of ANG II AT_1 receptors reduced the diabetes-induced glomerular hyperfiltration, indicating increased AT_1 receptor-mediated efferent arteriolar vasoconstriction. Augmented GFR in early diabetic nephropathy has been attributed to afferent arteriole dilation and an associated increase in RBF (7). However, we observed no differences in RBF between control and diabetic animals. This divergence of results has previously been attributed to insulin replacement (7, 42). Unaltered RBF has been reported not only in insulin-treated diabetic animals but also in response to salt load (10, 23). Diabetic hyperfiltration is associated with increased Na^+/glucose cotransport (52). In the present study, diabetics had increased T_{Na} as reported previously (55), which is associated with increased Q_{O_2} (19). Interestingly, adenosine A_1 receptor blockade had the same effect observed in controls, which also suggests a more distal regulation of T_{Na} in diabetics. As opposed to controls, where ANG II seems to influence T_{Na} in different parts of the nephron, in diabetics it mainly increased Na^+ transport in more proximal segments of the tubule, since FE_{Li} increased after infusion of candesartan or the combination of both antagonists. Further analyses are needed to accurately determine the major site of ANG II AT_1 receptor-mediated T_{Na} in diabetics, although it seems that in diabetics it is shifted to the proximal tubule or/and Loop of Henle. It might be speculated that increased proximal tubular reabsorption in diabetes, via enhanced Na^+/glucose contransport and enhanced peritubular capillary uptake force due to the increased FF, may reduce the hydrostatic pressure in the Bowman's capsule. This would directly increase the net filtration pressure and thus favor glomerular hyperfiltration (39). If the objective of therapy in early type 1 diabetes is to combat hyperfiltration, and to reduce arterial pressure and oxidative stress, the use of an adenosine A_1 receptor antagonist alone, or in combination with ANG II AT_1 receptor blockade, does not add anything to the standard-of-care therapy with an ANG II AT_1 receptor blockade because only candesartan reduced GFR and this was not augmented by adding DCPCX. The present study also demonstrates that tonic activation of ANG II AT_2 or adenosine A_2 receptors has no major roles in diabetes.

Hyperglycemia has been shown to increase intrarenal ANG II levels in rats (31), and ANG II AT_1 receptor activation is a key mediator of the increased oxidative stress in the diabetic kidney by direct induction of superoxide radical production by the NADPH oxidase (30). Nitric oxide (NO) bioavailability is decreased already in early stages of diabetic nephropathy (4, 34) and ANG II AT_1 receptor blockade restores NO levels in the diabetic kidney (4). It is therefore possible that the observed vasodilatation after ANG II AT_1 receptor blockade is at least partly due to increased NO bioavailability. On the other hand, in control animals, the renal vasculature is more sensitive to adenosine-mediated vasoconstriction when NO production is inhibited (5). Similarly, the streptozotocin-diabetic rat kidney is more prone to adenosine A_1 receptor-mediated vasoconstriction (41), which has been ascribed to decreased NO-dependent vasodilatation of the afferent arteriole (40).

AT_1 receptor blockade increased urine flow and FE_{Na} but did not alter FE_{Li} and T_{Na} in controls. In fact, activation of ANG II AT_1 receptors increases T_{Na} in both proximal and distal tubular segments and in collecting ducts (18). ANG II AT_2 receptors have been shown to contribute to proximal tubule Na^+ reabsorption (11), and the superimposed antagonism of ANG II AT_2 receptors to that of ANG II AT_1 receptors resulted in unaffected FE_{Na} and T_{Na}, which suggests that ANG II-mediated tubular Na^+ transport might occur in distal parts of the nephron. Blockade of adenosine A_1 receptors in controls increased urine flow, FE_{Na}, and T_{Na}, which is in good agreement with published results (53). Surprisingly, FE_{Li} was not significantly elevated by DPCPX (57). Although Li^+ has been widely used as an indicator of proximal tubule reabsorption in cases of low FE_{Na}, as observed in the present report, Li^+ may also be reabsorbed in more distal parts of the nephron (16), possibly in the loop of Henle (9, 47). Moreover, most of the previous studies investigating tubular function utilize state of the art micropuncture techniques (26, 43, 57). The nature of this technique only allows investigation of single superficial nephrons (51). Although further studies are needed to fully explain the discrepancies, our results suggest that tonic baseline activation of adenosine A_1 receptors regulates T_{Na} in distal parts of the nephron. Superimposed adenosine A_2 receptor antagonism to that of adenosine A_1 receptor blockade reduced urine flow and decreased FE_{Na}. This finding supports a role for in vivo tonic activation of adenosine A_2 receptors in mediating diuresis and natriuresis (58), at least when adenosine A_1 receptor function is reduced. Simultaneous blockade of ANG II AT_1 and adenosine A_1 receptors in controls also increased urine flow and FE_{Na}, although T_{Na} and Q_{O_2} increased. The most likely explanation for these seemingly contradictory results is that increased tubular Na^+ load is due to the increased GFR. Increased Na^+ in the macula densa activates TGF by stimulating adenosine production, which activates A_1 receptors in the afferent arteriole causing vasoconstriction and normalization of single nephron GFR (46, 53). In the present study, A_1 receptor blockade elicited a greater increase in FE_{Na} compared with that after blockade of ANG II AT_1 receptors. The concomitant increase in GFR together with increased FE_{Na} after adenosine A_1 receptor blockade implies TGF inactivation.

In conclusion, both ANG II AT_1 receptors and adenosine A_1 receptors are tonically active in control and diabetic animals during baseline and significantly influence intrarenal hemodynamics and tubular function. The hemodynamic effects mediated by simultaneous blockade of both receptors are not additive, indicating that ANG II and adenosine regulate intrarenal

hemodynamics via a unifying pathway that requires activation of both receptor types. However, this study demonstrates that increased AT_1 receptor signaling, independent of adenosine A_1 receptors, is a mechanistic explanation for diabetes-induced glomerular hyperfiltration. Finally, both ANG II AT_1 receptors and adenosine A_1 receptors tonically influence tubular Na^+ reabsorption in both control and diabetic animals, but the ANG II-mediated effects seem to be altered in the diabetic kidney. The clinical relevance derives from the finding that adenosine A_1 receptor blockade alone, or in combination with ANG II AT_1 receptor blockade, does not add anything to the standard-of-care therapy with an ANG II AT_1 receptor blockade if the objective is to reduce glomerular hyperfiltration and arterial pressure in early type 1 diabetes.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: D. Patinha, M.M., and F.P. conception and design of research; D. Patinha and A.F. performed experiments; D. Patinha, A.F., D. Pinho, M.M., and F.P. analyzed data; D. Patinha, M.M., and F.P. interpreted results of experiments; D. Patinha prepared figures; D. Patinha drafted manuscript; D. Patinha, A.A.-T., M.M., and F.P. edited and revised manuscript; D. Patinha, A.F., D. Pinho, A.A.-T., M.M., and F.P. approved final version of manuscript.

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TRABALHO 2

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Interrelationship between renin-angiotensin-aldosterone system and oxidative stress in chronic heart failure patients with or without renal impairment

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ABSTRACT

We investigated oxidative stress and RAAS biomarkers, as well as their association, in chronic heart failure (CHF) patients on optimized medical therapy, stratified by disease severity or by renal function. Since vitamin D has been shown to attenuate RAAS activation and oxidative stress, we further evaluated the relationship between vitamin D, RAAS and oxidative stress in CHF patients with or without renal impairment. Sixty CHF outpatients were included and stratified by disease severity or by renal function. We quantified urinary hydrogen peroxide, plasma and urinary isoprostanes, plasma total antioxidant status, urinary angiotensinogen (intrarenal RAAS activation biomarker) and plasma angiotensinogen, plasma renin and aldosterone concentration, serum angiotensin-converting enzyme (ACE) activity, plasma angiotensin peptides, and serum total 25-hydroxyvitamin D (S-total 25(OH)D). Severe CHF patients had higher urinary isoprostanes ($p = 0.002$) and lower S-total 25(OH)D ($p = 0.006$) compared to mild-to-moderate patients, but no differences were observed for other redox or RAAS biomarkers. Patients with impaired renal function (iRF) had higher urinary angiotensinogen ($p = 0.003$) and

Abbreviations: ACE, angiotensin-converting enzyme; ACEi, angiotensin-converting enzyme inhibitors; AGT, angiotensinogen; AKI, acute kidney injury; Ang I, angiotensin I; Ang II, angiotensin II; BHT, butylated hydroxy toluene; BNP, B-type natriuretic peptide; CHF, chronic heart failure; CRS, cardiorenal syndrome; eGFR, estimated glomerular filtration rate; ELISA, enzyme-linked immunosorbent assay; FAPGG, N-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine; H_2O_2, hydrogen peroxide; HF, heart failure; iRF, impaired renal function; LVSD, left ventricular systolic dysfunction; MR, mineralocorticoid receptor; nRF, normal renal function; NYHA, New York Heart Association; S-ACE, serum angiotensin-converting enzyme activity; P-AGT, plasma angiotensinogen; P-Ang I, plasma angiotensin I; P-Ang II, plasma angiotensin II; P-Ang(1-7), plasma angiotensin 1-7; P-Isop, plasma isoprostanes; P-renin, plasma renin concentration; P-TAS, plasma total antioxidant status; RAAS, renin-angiotensin-aldosterone system; ROS, reactive oxygen species; P-Aldost, plasma aldosterone concentration; SEM, standard error of the mean; S-total 25(OH)D, serum total hydroxyvitamin D; U-AGT, urinary angiotensinogen; U-H_2O_2, urinary hydrogen peroxide; U-Isop, urinary isoprostanes.

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lower S-total 25(OH)D ($p = 0.028$) compared to those with normal renal function (nRF), while no differences were observed for the remaining RAAS and redox parameters. Several positive correlations between oxidative stress and RAAS biomarkers were detected in iRF patients, while in patients with nRF these correlations were primarily inverse. In CHF-iRF patients, S-25(OD)D was inversely associated with urinary isoprostanes, which in turn were positively associated with plasma angiotensinogen and serum ACE. In conclusion, CHF patients with renal function impairment have increased intrarenal RAAS activation and lower vitamin D values and might benefit from the combination of RAAS blockers with vitamin D and/or antioxidants.

1. Introduction

The coexistence of kidney and heart failure (HF) is increasing and represents a major clinical challenge that is nowadays defined as the cardiorenal syndrome (CRS) [1]. Its pathogenesis is complex and yet not well understood, but is associated with adverse prognosis [2].

It is well established that systemic and renal oxidative stress can contribute to CRS pathology, particularly in CRS type 2 [3,4]. Among reactive oxygen species (ROS), hydrogen peroxide (H_2O_2) appears to be particularly relevant in the context of CRS as it is generated by various enzymes in the heart and kidney, exerts both protective and deleterious effects in the heart and contributes to renal injury [5–11].

Activation of the renin-angiotensin-aldosterone system (RAAS) is also widely recognized as an important neurohumoral mechanism in HF, being involved in both cardiac and renal dysfunction [12,13]. Moreover, independently from the systemic RAAS, activation of local tissue system also plays a role in the pathophysiology of renal disease and HF [14,15]. In this context, the urinary excretion of angiotensinogen (AGT) appears to be particularly relevant as it is considered an index of intrarenal RAAS activity and has been associated with renal dysfunction and arterial hypertension [16,17]. Furthermore, it has also been suggested as a prognostic marker in renal diseases and in acute decompensated HF [18–20].

RAAS and oxidative stress are known to be interrelated. It has been extensively demonstrated that angiotensin II (Ang II) stimulates ROS generation by NADPH oxidases and other prooxidant enzymes, thus contributing to cardiovascular and renal dysfunction [21–24]. Recent evidence supports that chronic RAAS activation can increase oxidative stress, leading to renal injury and to a vicious cycle of more sodium and water retention in CRS type 2 [3]. However, although angiotensin-converting enzyme inhibitors (ACEi) are a first-line treatment in HF [25], even in the presence of renal dysfunction [26], they seem to have little benefit in the setting of existing CRS type 2 [27]. Interestingly, accumulating evidence indicates that ROS also regulate RAAS activation [7,23,28], although evidence comes mostly from experimental studies concerning the kidney, where ROS were shown to increase AGT, renin, angiotensin-converting enzyme (ACE), Ang II, mineralocorticoid receptor (MR) and aldosterone [28]. Thus, it is possible that the lack of significant benefits from ACEi therapy in the setting of CRS type2 [27] results from its inability to effectively target the self-perpetuating cycle of RAAS-oxidative stress-renal dysfunction. Of note, there is evidence suggesting that renal impairment might be a major factor determining the relevance of therapies targeting oxidative stress in HF. While antioxidant treatment was shown to reduce cardiovascular morbidity and mortality in patients with end-stage renal disease, it did not exert a significant impact in patients at high-risk for cardiovascular events but with normal or slightly altered renal function [29,30].

Few studies evaluated both oxidative stress and RAAS activation biomarkers in patients with chronic HF (CHF) under optimized medical therapy [31,32]. This is probably due to the anticipated difficulties in interpreting results in patients receiving a combination of several medications that may interfere with both pathways [33].

We aimed to characterize, comprehensively, oxidative stress and RAAS in patients with CHF under optimized medical therapy, stratified by disease severity or by renal function. Newly, we looked at H_2O_2, AGT and several angiotensin peptides, which have been scarcely explored in human CHF and CRS, although we also measured commonly used markers of oxidative stress (e.g. isoprostanes, total antioxidant status) and RAAS (e.g. plasma renin, ACE activity, aldosterone) [34–39]. Since renal disease is associated with insufficiency or deficiency of vitamin D [40,41], which has been shown to exert protective effects against oxidative stress and RAAS over-activation [42–45], we also investigated the relationship between vitamin D status, oxidative stress and RAAS parameters in CHF patients with or without renal dysfunction.

2. Methods

2.1. Study population

Outpatients ($n = 60$) with an established HF diagnosis (New York Heart Association (NYHA) classes I to IV) for at least 2 years, with left ventricular systolic dysfunction (LVSD) assessed by echocardiography and optimized medical therapy, were recruited from the HF clinic of Centro Hospitalar São João, Porto, Portugal. We excluded patients with history of acute coronary syndrome, any hospital admission, therapeutic adjustment or infection, in the previous 3 months. All eligible patients provided written informed consent to participate in the study. This study was conducted in accordance with the Guidelines for Good Clinical Practice and the 1975 Declaration of Helsinki after approval by the institution's Ethics Committee.

2.2. Data collection

All patients were asked to collect a 24-h urine sample (8 AM to 8 AM the next morning) along the day before the visit to the HF clinic of Centro Hospitalar São João. On the day of the visit, the patients brought the 24h-urine samples to the clinic and fasting venous blood samples were collected between 8 AM and 11 AM. A physical examination was performed to all patients, and a record of demographic data, comorbidities, and medication in use was completed. To prevent possible bias concerning the clinical evaluation and NYHA class assignment, the same physician examined all the patients. However, we cannot exclude bias from patients' subjective self-assessment of symptoms and physical limitations.

CHF patients were stratified by disease severity or by the presence or absence of CRS defined here as impaired renal function (iRF) (estimated glomerular filtration rate, eGFR < 60 mL/min per 1.73 m^2) or normal renal function (nRF) (eGFR > 60 mL/min per 1.73 m^2), respectively. [46]

2.3. Laboratory procedures

All the routine laboratory analyses were performed at the Clinical Pathology Department of CHSJ. Plasma B-type natriuretic peptide (BNP) and troponin I were quantified by chemiluminescent microparticle immunoassays using an Abbot® Architect i2000 automated analyser (Abbott® Diagnostics, Lake Forest, IL, USA). An Olympus AU5400, Beckman Coulter® automated clinical chemistry analyser (Olympus, Hamburg, Germany) was used for the quantification of serum urea concentration by a kinetic urease/glutamate dehydrogenase method, plasma and urine creatinine by the colorimetric Jaffe method and

urinary protein by the pyrogallol red method. Urinary sodium was measured by an ion-selective electrode method. Plasma renin concentration (P-renin) and plasma aldosterone concentration (P-aldosterone) were measured by a radioimmunoassay and serum ACE activity (S-ACE) was quantified in an Olympus AU5400, Beckman Coulter® automated clinical chemistry analyser (Olympus, Hamburg, Germany) by a spectrophotometric method using N-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine (FAPGG) as substrate (Bühlmann®, Bühlmann Laboratories AG, Schönenbuch, Switzerland). Vitamin D status was evaluated by measuring serum total 25-hydroxyvitamin D (S-total 25 (OH)D) [47] by an electrochemiluminescent immunoassay using a Cobas E411 automated analyser and the Elecsys® Vitamin D total kit (Roche Diagnostics GmbH, Mannheim, Germany). All the samples used for the determination of other RAAS (AGT and angiotensin peptides) or oxidative stress biomarkers were processed at the Department of Biomedicine – Unit of Pharmacology and Therapeutics, Faculty of Medicine of Porto, Portugal, and stored at -80 °C until assayed.

2.4. Quantification of AGT and angiotensin peptides

AGT was quantified in plasma (P-AGT) and spot urine samples (U-AGT) by an enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Human Total Angiotensinogen Assay Kit, Immuno-Biological Laboratories Co., Gunma, Japan) accordingly to the protocol provided by the manufacturer.

Angiotensin I (Ang I), angiotensin II (Ang II) and angiotensin 1-7 (Ang 1-7) peptides were extracted from plasma samples using Sep-Pack-C18 columns (Discovery® DSC-Ph SPE, Supelco by Sigma-Aldrich/Merck, Sintra, Portugal) and then, the plasma values of Ang I (P-Ang I), Ang II (P-Ang II) and Ang 1-7 (P-Ang 1-7) were quantified by reverse phase high-performance liquid chromatography with UV detection [48]. Peaks of interest were identified by retention time of peptide standards and the results expressed in pg/mL.

2.5. Quantification of oxidative stress biomarkers

H_2O_2 was quantified in 24h-urine samples (U-H_2O_2) by a microplate fluorimetric assay (Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit; Molecular Probes, Invitrogen, Thermo Fisher Scientific, Massachusetts, USA). Urinary isoprostanes (U-Isop) were quantified in 24h-urine samples by a competitive enzyme immunoassay (Urinary Isoprostane ELISA Kit; Oxford Biomedical Research® Inc., Oxford, MI, USA) in non-extracted urine containing the preservative butylated hydroxy toluene (BHT, 0.005 %, w/v) added before storage, and incubated with β-glucuronidase before the assay. Total isoprostanes were quantified in plasma (P-Isop) containing the preservatives BHT (0.005 %, w/v) and indomethacin (10 μM), which were added before storage. Hydrolysis of sterified isoprostanes and solid-phase extraction (using Sep-Pak Classic C18 and Sep Pak Vac-Silica columns, Waters® Corporation, Milford, MA, USA) were performed before the measurement of P-Isop by a competitive enzyme immunoassay (15-Isoprostane F2t ELISA Kit; Oxford Biomedical Research® Inc., Oxford, MI, USA), according to the protocol provided by the manufacturer. Plasma total antioxidant status (P-TAS) was evaluated by a spectrophotometric assay (Antioxidant Assay Kit; Cayman Chemical Company, Ann Arbor, MI, USA) that measures the combined antioxidant activities of water- and lipid soluble antioxidants, including vitamins, glutathione, uric acid, bilirubin, albumin, etc. The antioxidant capacity of the sample is compared with that of Trolox, a water-soluble tocopherol analogue, and is expressed as mM Trolox equivalents.

2.6. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM) or as median (25th percentile; 75th percentile), or as percentage, and represented graphically as scatter plots. eGFR was determined using the Cockroft–Gault formula given its low cost and universal availability. [49]

Statistical analysis was performed using unpaired Student's *t*-test or Mann–Whitney U-test, where appropriate. Categorical variables were analysed by the Fisher's exact test. *P*-values of <0.05 were considered significant. For correlation analysis, a base 10 log transformation was applied to all variables with non-normal distribution. Pearson's single regression or Spearman's correlation analyses were used to estimate correlations between sets of parametric or nonparametric data, respectively. Multivariate linear regression was applied to determine the relationship between RAAS biomarkers and oxidative stress, between RAAS biomarkers and S-total 25(OH)D or between oxidative stress and S-total 25(OH)D, adjusted for some confounders namely age, gender and BMI. Only variables that presented significant correlations in Pearson's or Spearman's analyses were evaluated in the multivariate linear regression models. Coefficient regression (β), 95 % confidence intervals (95 % CI), *P*-value and coefficient of determination (R^2) were presented. The results were stratified according to renal function (eGFR<60 mL/min per 1.73 m^2 or eGRF ≥60 mL/min per 1.73 m^2). There were missing values in some biomarkers due to insufficient sample volume of samples or reagents to perform sample processing, dilution tests and assays. The final number per group for these parameters was as following: troponin I, n = 34 vs 24 (mild-to-moderate CHF vs severe CHF) and n = 22 vs 36 (nRF vs iRF); urea, n = 35 vs 24 (mild-to-moderate CHF vs severe CHF) and n = 24 vs 35 (nRF vs iRF); proteinuria, n = 33 vs 22 (mild-to-moderate CHF vs severe CHF) and n = 22 vs 33 (nRF vs iRF); U-H_2O_2 and U-Isop, n = 35 vs 24 (mild-to-moderate CHF vs severe CHF); n = 23 vs 36 (nRF vs iRF); P-Isop, n = 33 vs 23 (mild-to-moderate CHF vs severe CHF); n = 20 vs 36 (nRF vs iRF); P-TAS, n = 33 vs 22 (mild-to-moderate CHF vs severe CHF) and n = 19 vs 35 (nRF vs iRF), P-renin, n = 30 vs 21 (mild-to-moderate CHF vs severe CHF) and n = 19 vs 32 (nRF vs iRF), P-Aldost, n = 33 vs 21 (mild-to-moderate CHF) and n = 21 vs 33 (nRF vs iRF); S-ACE, n = 29 vs 24 (mild-to-moderate CHF vs severe CHF) and n = 22 vs 31 (nRF vs iRF); P-AGT, n = 33 vs 20 (mild-to-moderate CHF vs severe CHF) and n = 20 vs 33 (nRF vs iRF); U-AGT, n = 35 vs 19 (mild-to-moderate CHF vs severe CHF) and n = 21 vs 32 (nRF vs iRF); P-Ang I, P-Ang II and P-Ang 1−7, n = 30 vs 13 (mild-to-moderate CHF vs severe CHF) and n = 17 vs 26 (nRF vs iRF); S-total 25(OH)D, n = 32 vs 21 (mild-to-moderate CHF vs severe CHF) and n = 20 vs 31 (nRF vs iRF).

Based on the values described by Chen et al. [19] for U-AGT values in patients with acute decompensated HF with or without progressed acute kidney injury, we found that a sample size of 19 patients in each group would be sufficient to obtain a statistical study power of 80 % at a 5% level of significance. We first used the formulas provided by Wan et al. [50] to estimate the sample mean and standard deviation from the sample size, median and 25th and 75thpercentiles values described by Chen et al. [19] and further calculated sample size using G*Power 3.1.

3. Results

3.1. Demographical, clinical and biochemical characteristics

Demographic and clinical characteristics of the study population are displayed in Table 1.

There were 36 patients with mild-to-moderate CHF (NYHA stages I and II) and 24 patients with severe CHF (NYHA stages III and IV) stratified by disease severity. Mild-to-moderate CHF patients were predominantly men and significantly younger and heavier than severe CHF patients. HF etiologies were similar among the groups, with ischaemic heart disease being the predominant cause of HF. Severe CHF group included significantly more patients receiving nitrates and less patients being treated with a RAAS inhibitor compared to the mild-to-moderate CHF group. No significant differences were observed for the other treatments. Severe CHF patients had significantly higher concentrations of BNP, troponin I, urea and lower values of eGFR.

When patients were stratified according to renal function, there were

Table 1
Demographic, clinical and biochemical parameters of the study population stratified by CHF severity or by renal function.

Demographic, clinical and biochemical parameters	Mild-to-moderate CHF (n = 36)	Severe CHF (n = 24)	*P* value	CHF patients with normal renal function (nRF) (eGFR ≥ 60 mL/min per 1.73 m^2) (n = 24)	CHF patients with impaired renal function (iRF) (eGFR < 60 mL/minper 1.73 m^2) (n = 36)	*P* value
NYHA class I, n (%)	16 (44)	—	—	10 (42)	6 (17)	0.0410
NYHA class II, n (%)	20 (56)	—	—	9 (38)	11 (31)	0.5846
NYHA class III n (%)	—	7 (29)	—	2 (8)	5 (14)	0.6913
NYHA class IV, n (%)	—	17 (71)	—	3 (13)	14 (39)	0.0399
Age (years)	**67 ± 2**	**75 ± 2**	**0.0169**	**60 ± 2**	**76 ± 1**	**<0.0001**
Gender*: Men, n (%)	**27 (75)**	**11 (46)**	**0.0299**	19 (79)	19 (53)	0.0557
Gender*: Women, n (%)	**9 (25)**	**13 (54)**	**0.0299**	5 (21)	17 (47)	0.0557
Weight (kg)	**76 ± 2**	**67 ± 2**	**0.0180**	**79 ± 3**	**69 ± 2**	**0.0047**
BMI (kg/m^2)	28 ± 1	26 ± 1	0.1153	**29 ± 1**	**26 ± 1**	**0.0417**
SBP (mmHg)	123 ± 3	114 ± 4	0.0990	119 ± 4	119 ± 4	0.9762
DBP (mmHg)	66 (60; 78)	68 (60; 70)	0.4724	68 (60; 78)	65 (60; 73)	0.3088
HR (beat/min)	72 ± 2	67 ± 2	0.0960	74 ± 3	68 ± 1	0.0638
Etiology						
Ischemic, n (%)	14 (39)	11 (46)	0.6056	**6 (25)**	**19 (53)**	**0.0375**
Hypertensive, n (%)	8 (22)	5 (21)	>0.9999	4 (17)	9 (25)	0.5336
Alcoholic cardiomyopathy, n(%)	6 (17)	4 (17)	>0.9999	**9 (38)**	**1 (3)**	**0.0007**
Valvular heart disease, n (%)	1 (3)	2 (8)	0.5746	1 (4)	2 (6)	>0.9999
Idiopatic cardiomyopathy/ Unknown, n (%)	7 (19)	3 (13)	0.3337	3 (13)	7 (19)	0.7255
Other, n (%)	1 (3)	1 (4)	>0.9999	2 (8)	0 (0)	0.1559
Treatment						
ACE inhibitor and/or ARB blocker, n (%)	**36 (100)**	**17 (71)**	**0.0009**	23 (96)	30 (83)	0.2254
Beta-blocker, n (%)	30 (83)	19 (79)	0.7412	19 (79)	30 (83)	0.7412
Loop diuretic, n (%)	32 (89)	24 (100)	0.1412	23 (96)	33 (92)	0.6434
Nitrates, n (%)	**3 (8)**	**8 (33)**	**0.0197**	**1 (4)**	**10 (28)**	**0.0375**
Biochemical parameters/ biomarkers						
BNP (pg/mL)	**215 (153;399)**	**822(337; 2013)**	**0.0003**	**185 (137;275)**	**504 (256;1685)**	**0.0002**
Troponin I (ng/mL)	**0.014 (0.005; 0.029)**	**0.032 (0.010;0.071)**	**0.0446**	0.011 (0.005;0.028)	0.025 (0.005;0.049)	0.1111
Urea (mmol/L)	**9.3 (6.8; 11.5)**	**12.9 (8.6; 19.5)**	**0.0112**	**8.5 (6.4; 10.1)**	**12.8 (8.8; 18.3)**	**0.0004**
eGFR (mL/min per 1.73 m^2).	**66.0 ± 4.6**	**45.3 ± 4.3**	**0.0027**	**83.8 ± 4.3**	**40.3 ± 2.0**	**<0.0001**
Proteinuria (g/day)	0.18 (0.15;0.22)	0.15 (0.11;0.21)	0.1360	0.17 (0.14;0.22)	0.17 (0.12;0.22)	0.5461
U-Na^+ (mmol/mg creat.)	0.13 (0.10; 0.18)	0.17 (0.14;0.23)	0.0566	0.15 (0.11; 0.20)	0.14 (0.10;0.19)	0.3640

ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker; BMI, body mass index; BNP, B-type natriuretic peptide; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; HR, heart rate; NYHA, New York Heart Association; SBP, systolic blood pressure; U-Na^+, urinary excretion of sodium. Results are expressed as number (%), mean ± SEM or as median (25th percentile; 75th percentile) for data with normal or non-normal distribution, respectively.
Bold values: statistically significant results between groups.

24 patients with normal renal function (nRF, eGFR ≥ 60 mL/min per 1.73 m^2) and 36 patients with impaired renal function (iRF, eGFR < 60 mL/min per 1.73 m^2). Patients with iRF were significantly older and with lower weight and body mass index (BMI), had predominantly HF of ischemic etiology and consequently included a significantly higher number of patients treated with nitrates. They also presented higher BNP and urea concentrations. Alcoholic cardiomyopathy was the predominant cause of HF in nRF patients.

3.2. RAAS biomarkers

Patients stratified according to disease severity did not present significant differences in RAAS biomarkers, namely P-renin, P-Aldost, S-ACE, P-AGT, U-AGT, P-Ang I, P-Ang II or P-Ang 1−7 (Table 2). However, when patients were stratified by renal function, those with iRF had significantly higher U-AGT values (more than a two-fold increase in median values, p = 0.003) than patients with nRF (Table 2).

3.3. Oxidative stress biomarkers

Patients with severe CHF had significantly higher U-Isop (p = 0.002) than mild-to-moderate CHF patients, but there were no significant differences for the other oxidative stress biomarkers. When patients were stratified by renal function, we did not observe differences in oxidative stress biomarkers between groups (Table 2).

3.4. Vitamin D status

Severe CHF patients had significantly lower values of S-total 25(OH)D compared to patients with mild-to-moderate CHF (p = 0.006). In patients stratified by renal function, those with iRF also presented significantly reduced S-total 25(OH)D compared to nRF patients (p = 0.028) (Table 2).

3.5. Correlation analysis

Patients with mild-to-moderate CHF exhibited significant inverse correlations between oxidative stress and RAAS markers (Log U-H_2O_2 vs P-Ang II; Log P-Isop vs Log P-AGT) (Fig. 1A and B, respectively), although there was a positive correlation of Log U-Isop with Log S-ACE (Fig. 1C). On the other hand, severe CHF patients only exhibited a significant positive correlation between Log U-H_2O_2 and Log P-renin (Fig. 1D). No correlation was observed between Log P-AGT and Log U-AGT.

When patients were stratified by renal function, the group with nRF presented inverse correlations between oxidative stress and RAAS markers, namely Log U-H_2O_2 vs P-Ang II (Fig. 2B) and Log P-Isop vs Log P-AGT) (Fig. 2C), as observed in the mild-to-moderate CHF group

Table 2
Oxidative stress, RAAS biomarkers and vitamin D status in the study population stratified by CHF severity or by renal function.

Oxidative stress and RAAS biomarkers	Mild-to-moderate CHF	Severe CHF	*P* value	CHF patients with normal renal function (nRF) (eGFR ≥ 60 mL/min per 1.73 m^2)	CHF patients with impaired renal function (iRF) (eGFR < 60 mL/min/ per 1.73 m^2)	*P* value
U-H_2O_2 (μmol/mg creat.)	0.009 (0.005;0.020)	0.012 (0.006;0.023)	0.483	0.011 (0.005;0.020)	0.011 (0.005;0.022)	>0.999
U-Isop (ng/mg creat.)	**2.2 (1.5;2.7)**	**3.2 (2.3;4.2)**	**0.002**	2.4 (1.8; 3.2)	2.6 (1.7; 3.5)	0.519
P-Isop (ng/mL)	0.6 (0.4;0.8)	0.8 (0.5;1.1)	0.065	0.7 (0.4;1.0)	0.6 (0.5;1.0)	0.803
P-TAS (mmol Trolox equiv./L)	2.4 (1.5;3.3)	1.5 (1.3;3.6)	0.873	2.2 (1.4; 3.4)	2.1 (1.4; 3.4)	0.918
P-AGT (μg/mL)	24 (19;29)	26 (19;36)	0.272	24 (16;35)	26 (20;30)	0.309
U-AGT (μg/g creat.)	16 (9;39)	29 (9;39)	0.502	**13 (7;20)**	**30 (13;64)**	**0.003**
P-Renin (μU/mL)	146 (29;260)	150 (56;682)	0.266	143 (64; 287)	151 (49;271)	0.900
P-Aldost (ng/dL)	9 (4;15)	14 (7;20)	0.131	9 (6;16)	12 (6;17)	0.789
S-ACE (U/L)	4 (2;7)	7 (2;33)	0.132	6 (4;10)	3 (2;22)	0.062
P-Ang I (pg/mL)	46.6 ± 2.1	46.8 ± 5.2	0.974	46.7 ± 2.2	46.6 ± 3.3	0.978
P-Ang II (pg/mL)	41.0 ± 2.9	35.9 ± 5.3	0.361	43.1 ± 4.3	37.0 ± 3.2	0.225
P-Ang 1−7 (pg/mL)	27.2 ± 3.5	33.1 ± 4.2	0.325	28.2 ± 4.2	29.5 ± 3.7	0.819
S-total 25 (OH) D (ng/mL)	**18 (11;24)**	**11 (6;17)**	**0.006**	**18 (12;25)**	**11 (6;18)**	**0.028**

P-AGT, plasma angiotensinogen; P-Aldost, plasma aldosterone; P-Ang I, plasma angiotensin I; P-Ang II, angiotensin II; P-Ang (1-7), plasma angiotensin 1-7; P-Isop, plasma isoprostanes; P-renin, plasma renin concentration; P-TAS, plasma total antioxidant status; S-ACE, serum angiotensin-converting enzyme activity; S-total 25 (OH)D, serum total 25-hydroxyvitamin D; U-AGT, urinary angiotensinogen; U-H_2O_2, urinary hydrogen peroxide; U-Isop, urinary isoprostanes. Results are expressed as mean ± SEM or as median (25th percentile; 75th percentile) for data with normal or non-normal distribution, respectively.
Bold values: statistically significant results between groups.

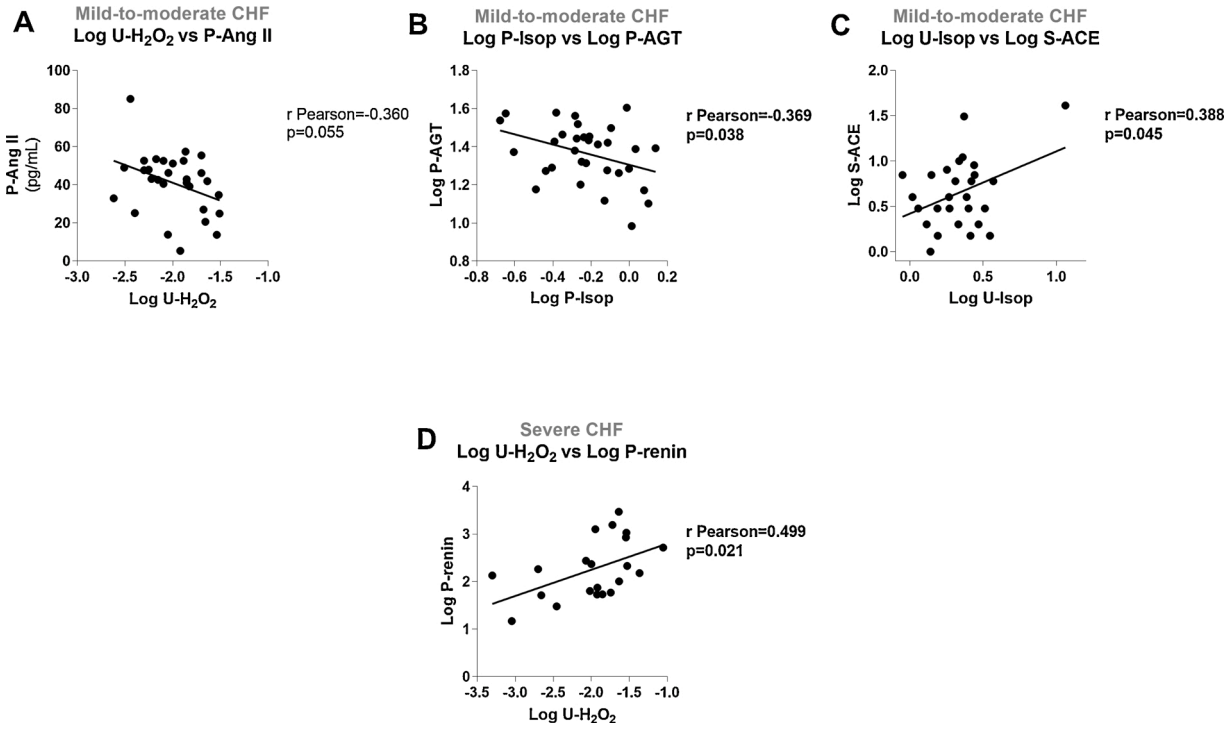

Fig. 1. Correlations between oxidative stress markers and RAAS parameters in mild-to-moderate and severe CHF: **(A)** Log U-H_2O_2 vs P-Ang II, mild-to-moderate CHF; **(B)** Log P-Isop vs Log P-AGT, mild-to-moderate CHF; **(C)** Log U-Isop vs Log S-ACE, mild-to-moderate CHF; (D) Log U-H_2O_2 vs Log P-renin, severe CHF.

(Fig. 1), but a positive correlation of Log U-H_2O_2 with P-Ang I (Fig. 2A). Differently, patients with iRF exhibited several significant positive correlations between oxidative stress and RAAS biomarkers, namely Log U-H_2O_2 vs Log P-renin (Fig. 3A), Log U-H_2O_2 vs Log P-AGT (Fig. 3B), Log U-Isop vs Log P-AGT (Fig. 3D) and Log U-Isop vs Log S-ACE (Fig. 3E) and a borderline significant positive correlation between Log U-H_2O_2 and Log U-AGT ($p = 0.057$) (Fig.3C). No correlation was observed between Log P-AGT and Log U-AGT.

In an attempt to identify factors that could be contributing to these correlations in CHF patients with nRF or with iRF, we also evaluated the correlations of S-total 25(OH)D with RAAS or oxidative stress biomarkers. We observed that S-total 25(OH)D values were positively correlated with P-Ang I in CHF-nRF patients (Fig. 4A), but not in CHF-iRF (Fig. 4B), and inversely correlated with U-Isop in CHF patients with iRF (Fig. 4D), but not in CHF-nRF (Fig. 4C).

3.6. Multivariate regression models

In the multivariate regression models adjusted for age, gender and BMI and stratified for eGFR, we observed that, in CHF patients with nRF,

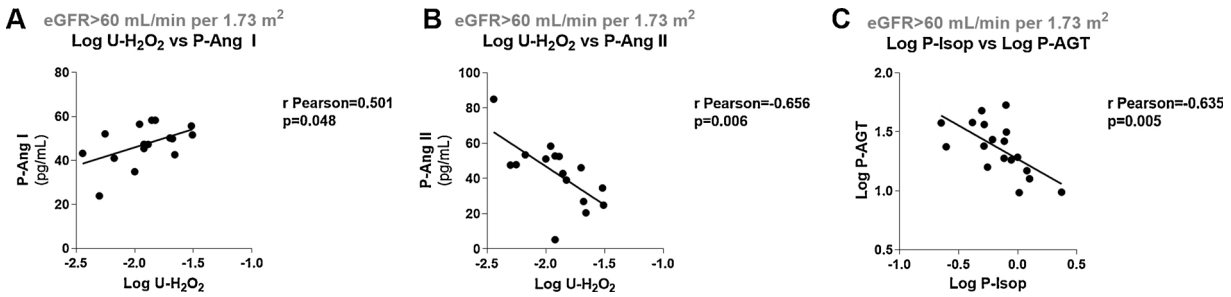

Fig. 2. Correlations between oxidative stress markers and RAAS parameters in CHF patients with nRF (eGFR>60 mL/min per 1.73 m^2): **(A)** Log U-H_2O_2 vs P-Ang I; **(B)** Log U-H_2O_2 vs P-Ang II; **(C)** Log P-Isop vs Log P-AGT.

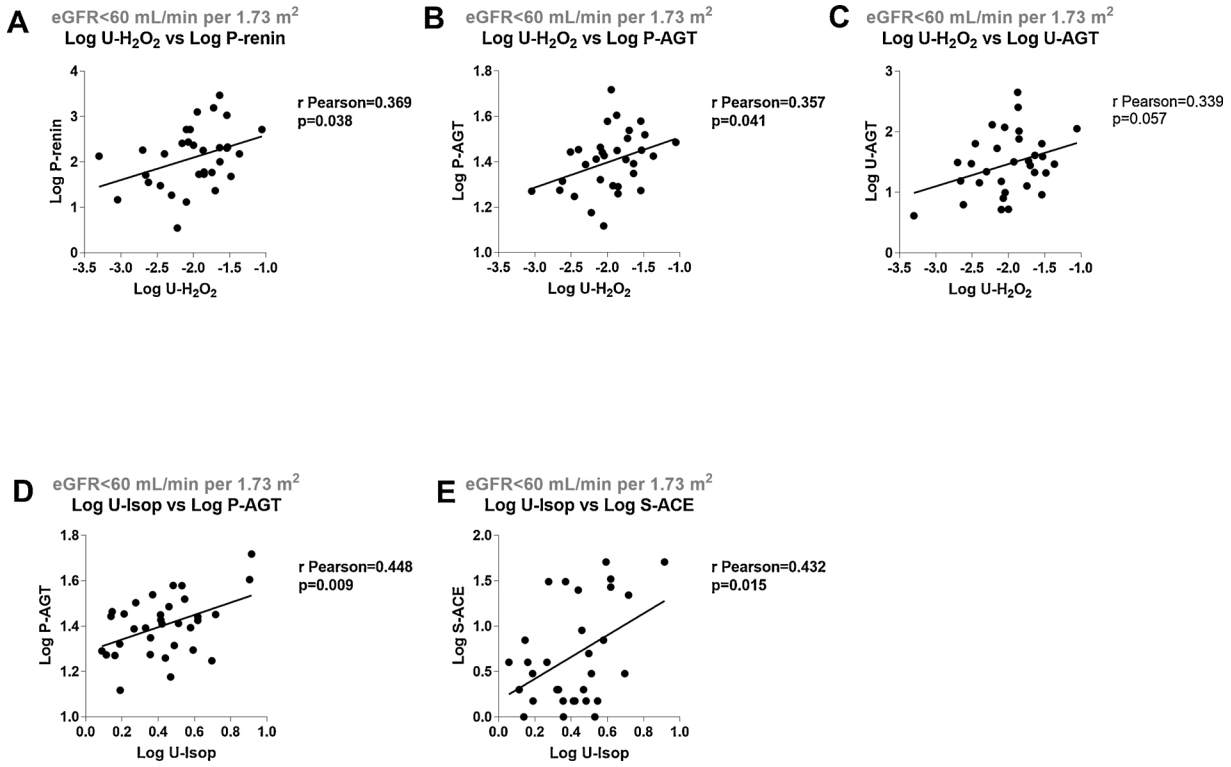

Fig. 3. Correlations between oxidative stress markers and RAAS parameters in CHF patients with iRF (eGFR< 60 mL/min per 1.73 m^2): **(A)** Log U-H_2O_2 vs P-renin; **(B)** Log U-H_2O_2 vs Log P-AGT; **(C)** Log U-H_2O_2 vs Log U-AGT; **(D)** Log U-Isop vs Log P-AGT; **(E)** Log U-Isop vs Log S-ACE.

the relationships between RAAS, oxidative stress and vitamin D status found in correlation analyses did not hold, although a borderline significant inverse association was shown for U-H_2O_2 and P-Ang II ($p = 0.068$) (Table 3).

In CHF patients with iRF, the relationships detected between U-Isop and RAAS markers or between vitamin D and U-Isop were confirmed in multivariate analysis, with U-Isop being associated with higher P-AGT ($R^2 = 0.46$) and S-ACE values ($R^2 = 0.54$), and S-total 25(OH)D being associated with lower U-Isop ($R^2 = 0.38$) (Table 4).

4. Discussion

Our study highlights the existence of several positive correlations between oxidative stress and RAAS biomarkers in CHF patients with iRF, while in CHF patients with nRF the correlations were primarily inverse. Also, we found higher concentration of U-AGT and lower values of serum total 25(OH)D in CHF patients with iRF compared to those with nRF.

U-AGT has been considered a biomarker of intrarenal RAAS modulation since AGT is locally produced in the S3 segment of the proximal tubule in higher extent than in the liver [51], and U-AGT levels are associated with increases in AGT expression and Ang II concentration in the kidney [17,52]. Concurrently, there is limited glomerular permeability to liver-derived plasma AGT and this has a minor contribution to U-AGT levels since it is mostly up taken from the urinary filtrate by megalin [51]. Under pathological conditions like severe podocyte injury, glomerular filtration of AGT of liver origin is increased by the loss of the macromolecular barrier function of the glomeruli [53]. Nevertheless, it has also been demonstrated that increased U-AGT precedes the onset of albuminuria in diabetic patients and is present in normo-albuminuric children with diabetes [54,55]. Also, in patients with chronic kidney disease, U-AGT values are increased and negatively associated with eGFR, independently of urinary albumin excretion [56]. Our CHF patients had similar levels of proteinuria, even when we stratified patients according to the presence of renal impairment. Furthermore, there were no significant correlations between P-AGT and U-AGT values, as it would be expected if U-AGT values resulted from filtration of P-AGT. Therefore, the increased U-AGT values observed in our study appear to mainly reflect intrarenal RAAS activation in CHF patients with iRF. U-AGT has been recently demonstrated to be an early

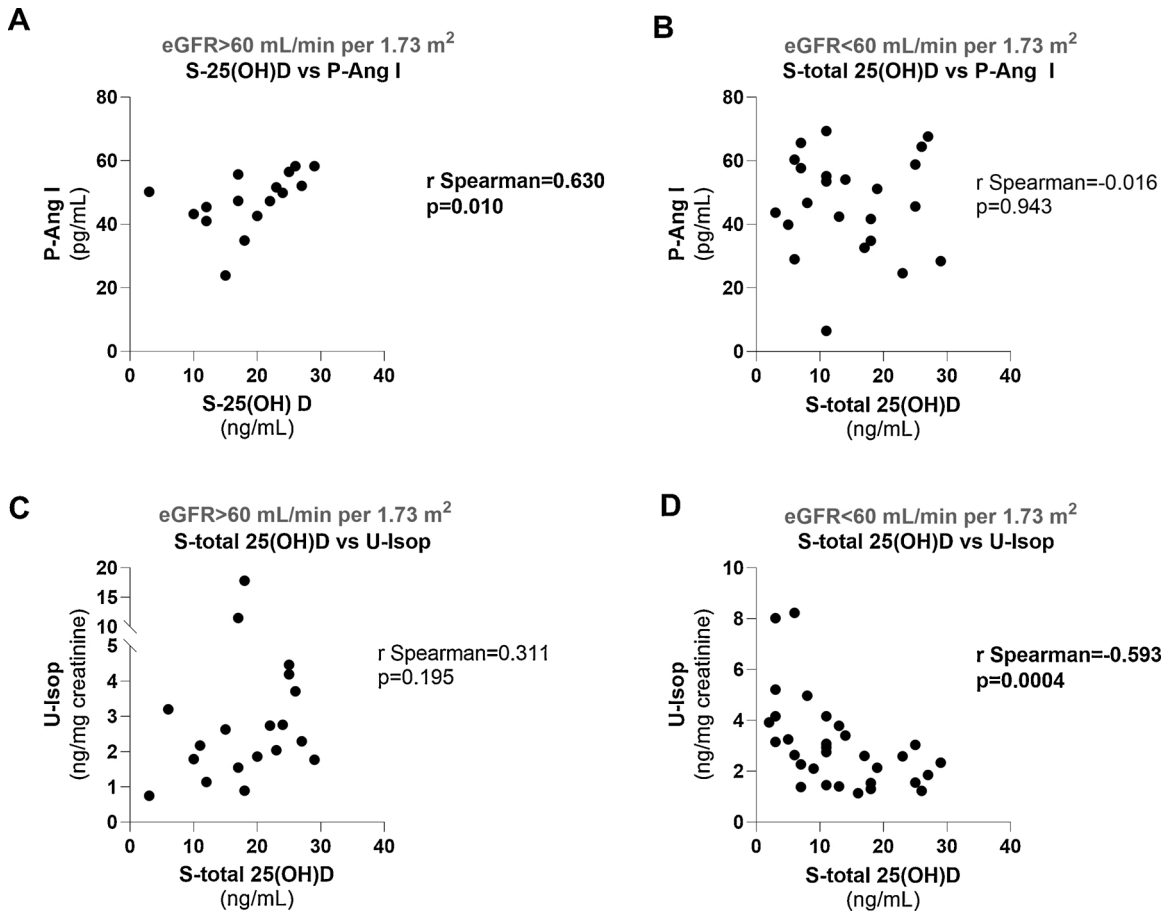

Fig. 4. Correlations of S-total 25(OH)D with P-Ang I or U-Isop in CHF patients with nRF (eGFR>60 mL/min per 1.73 m^2) or iRF (eGFR< 60 mL/min per 1.73 m^2): **(A, B)** S-total 25(OH)D vs P-Ang-I; **(C, D)** S-total 25(OH)D vs U-Isop.

predictor for acute CRS and 1-year prognosis in patients with acute decompensated HF [57]. When measured at the time of acute kidney injury diagnosis (AKI), U-AGT was also useful to predict AKI progression and to identify CRS patients with higher risk for adverse outcomes [19]. Thus, U-AGT appears to be an important biomarker for risk stratification in HF.

In our study, the lack of significant differences in other ROS and RAAS parameters between CHF patients with or without renal impairment, besides those observed in U-AGT, might be due to treatment with RAAS blockers and nitrates, which are known to influence both oxidative stress and RAAS markers [58–61].

The positive relationships found between urinary markers of oxidative stress and systemic RAAS parameters in CHF-iRF patients are not surprising. U-AGT is considered a marker of intrarenal AGT production [17,51,52], but U-H_2O_2 and U-Isop can reflect both systemic and renal oxidative stress [39,62–66] and systemic biomarkers of oxidative stress have been shown to correlate or associate with systemic RAAS [67–69]. Of note, a borderline significant positive correlation between U-H_2O_2 and U-AGT was also detected in patients with renal function impairment. This could mean that higher intrarenal H_2O_2 might be associated with intrarenal RAAS activation in these patients. The lack of a stronger correlation between these two biomarkers could be due to the small number of patients evaluated and to the fact that U-H_2O_2 does not solely reflect renal H_2O_2 production. Furthermore, some patients of this group were receiving nitrates which might decrease intrarenal RAAS activation and exert a protective effect on cardiorenal disease [58,61]. Interestingly, we observed that CHF-iRF patients treated with nitrates exhibited tendentially lower values of U-AGT than untreated CHF-iRF patients (Suppl Fig. 1H).

Experimental studies have shown that increased ROS bioavailability enhances RAAS activation, contributing to the development and progression of renal disease. In a model of diabetic nephropathy in Zucker diabetic fatty obese rats, an increase in U-Isop precedes the rise of renal AGT and Ang II concentrations, as well as of renal injury markers [70]. Also, in a mice model of IgA nephropathy, both ROS and RAAS activation contribute to renal injury in an early phase of the disease [71]. Furthermore, treatments with antioxidants have been shown to reduce the renal expression or urinary excretion of AGT in experimental models of arterial hypertension [23,72]. Noteworthy, our present results show, for the first time, that oxidative stress and RAAS biomarkers are positively correlated in CHF patients with renal impairment, while in CHF patients with nRF there were mainly inverse correlations between ROS and RAAS. This suggests that a positive feedback mechanism between ROS and RAAS, or vice-versa, operates in CRS, despite medical therapy, while in CHF patients with normal renal function, an effective negative feedback regulation between RAAS and ROS might be relevant to preclude renal impairment. Interestingly, we observed that, in patients with normal renal function, but not in patients with renal dysfunction, Ang II exhibited a borderline positive correlation with Ang 1-7 (Suppl Fig), which is known to counterregulate Ang II-induced ROS generation [73,74]. Thus, we speculate that Ang 1-7 production from Ang II might be an important factor contributing to the inverse relationships observed in CHF patients with normal renal function. Nevertheless, we cannot exclude other counterregulatory mechanisms beyond Ang 1−7 production, such as an inhibitory effect of H_2O_2 (or other ROS or ROS by-products) on the expression and/or activity of Ang II-producing enzymes [75,76], or a stimulatory effect on Ang II-degrading enzymes. In fact, ROS have been shown to either upregulate or downregulate the

Table 3
Multivariate linear regression models for RAAS markers (P-Ang I, P-Ang II and P-AGT) in CHF patients with eGFR≥60 mL/min per 1.73 m^2. (Coefficient regression (Adjusted β), 95 % confidence intervals (95 % CI), *P* value and coefficient of determination (R^2) estimated by linear regression models with P-Ang I, P-Ang II and P-AGT as the dependent variable and adjusted for age, gender and BMI).

eGFR ≥ 60 mL/min per 1.73 m^2				
	Adjusted β	95 % CI		*P* value
P-Ang I (pg/mL)				
Age (Years)	−0.105	−0.703	0.493	0.701
Gender (Male)	−8.834	−32.973	15.306	0.429
BMI (kg/m^2)	0.719	−0.596	2.034	0.247
U-H_2O_2 (μmol/mg creat)	312.356	−500.019	1124.731	0.407
R^2	0.54			
P-Ang II (pg/mL)				
Age (Years)	0.438	−0.409	1.284	0.272
Gender (Male)	−4.495	−38.665	29.675	0.773
BMI (Kg/m^2)	0.665	−1.196	2.527	0.440
U-H_2O_2 (μmol/mg creat)	−1055.904	−2205.825	94.016	0.068
R^2	0.50			
P-AGT (μg/mL)				
Age (Years)	0.387	−0.528	1.302	0.358
Gender (Male)	−6.922	−27.991	14.146	0.470
BMI (kg/m^2)	0.340	−1.481	2.161	0.678
P-Isop (ng/mL)	−25.999	−57.414	5.415	0.093
R^2	0.43			
P-Ang I (pg/mL)				
Age (Years)	−0.103	−0.961	0.754	0.791
Gender (Male)	−6.440	−34.592	21.711	0.617
BMI (kg/m^2)	0.855	−0.958	2.669	0.314
S-total 25(OH)D (ng/mL)	0.108	−1.304	1.519	0.867
R^2	0.31			

BMI, body mass index, P-AGT, plasma angiotensinogen; P-Ang I, angiotensin I; P-Ang II, plasma angiotensin II; P-Isop, plasma isoprostanes; S-total 25(OH)D, serum total 25-hydroxyvitamin D; U-AGT, urinary angiotensinogen; U-Isop, urinary isoprostanes.

expression and activity of RAAS parameters, depending on the physiological or pathological condition, tissues and species tested [28].

Renal disease is often associated with vitamin D insufficiency or deficiency [40,41]. In our study, CHF patients with impaired renal function had significantly lower values of 25(OH)D, a biomarker of vitamin D status, compared to patients with preserved renal function. Since vitamin D has been shown to reduce RAAS activation and oxidative stress [42–45], we investigated the correlations of serum 25(OH)D with RAAS and oxidative stress parameters in patients with or without renal impairment. Importantly, we observed that 25(OH)D was inversely correlated with U-Isop only in CHF-iRF patients and positively correlated with P-Ang I only in CHF-nRF. Furthermore, the inverse relationship between 25(OH)D and U-Isop, as well as the positive associations of U-Isop with P-AGT or S-ACE in CHF-iRF patients held in multivariate analysis controlled for age, gender and BMI. This suggests that markedly low vitamin D values in CHF-iRF patients contribute to enhanced oxidative stress that further aggravates RAAS activation, despite RAAS blockade. Indeed, a growing amount of experimental evidence and a recent meta-analysis of clinical trials have shown that vitamin D supplementation prevents or attenuates oxidative stress and associated damage through multiple mechanisms [42,45]. Therefore, the combination of RAAS blockers with vitamin D and/or antioxidants might be useful to block these escape mechanisms and prevent further cardiac and renal deterioration in CHF patients with renal dysfunction. Of note, vitamin D supplementation was shown to prevent the decline in eGFR in CKD patients using RAAS blockers [77]. Also, more recently, the authors of a study in coronary artery disease patients undergoing percutaneous coronary intervention underlined the need of conducting larger studies to assess the effect of vitamin D supplementation on RAAS

Table 4
Multivariate linear regression models for RAAS (P-Renin, P-AGT, U-AGT, S-ACE) and oxidative stress (U-Isop) markers in CHF patients with eGFR<60 mL/min per 1.73m^2. (Coefficient regression (Adjusted β), 95 % confidence intervals (95 % CI), *P* value and coefficient of determination (R^2) estimated by linear regression models with P-Renin, P-AGT, U-AGT, S-ACE or U-Isop as the dependent variable and adjusted for age, gender and BMI.).

eGFR <60 mL/min per 1.73 m^2				
	Adjusted β	95 % CI		*P* value
P-Renin (μU/mL)				
Age (Years)	−10.544	−50.942	29.855	0.592
Gender (Male)	397.046	−179.969	974.060	0.167
BMI (kg/m2)	−9.537	−77.903	58.828	0.774
U-H_2O_2 (μmol/mg creat)	4494.721	−11804.430	20793.872	0.572
R^2	0.15			
P-AGT (μg/mL)				
Age (Years)	−0.466	−0.988	0.056	0.077
Gender (Male)	−1.287	−8.640	6.067	0.719
BMI (kg/m^2)	0.200	−0.651	1.051	0.629
U-H_2O_2 (μmol/mg creat)	137.674	−69.067	344.416	0.180
R^2	0.21			
U-AGT (μg/g creat)				
Age (Years)	−0.002	−2.315	2.311	0.999
Gender (Male)	17.285	−16.075	50.646	0.292
BMI (kg/m^2)	−2.417	−6.339	1.505	0.213
U-H_2O_2 (μmol/mg creat)	407.749	−506.935	1322.432	0.363
R^2	0.19			
P-AGT (μg/mL)				
Age (Years)	−0.399	−0.831	0.034	0.069
Gender (Male)	0.769	−5.457	6.995	0.799
BMI (kg/m^2)	0.249	−0.435	0.933	0.456
U-Isop (ng/mg creat)	**3.260**	**1.307**	**5.214**	**0.002**
R^2	0.46			
S-ACE (U/L)				
Age (Years)	−0.150	−0.777	0.477	0.624
Gender (Male)	3.685	−4.879	12.248	0.381
BMI (kg/m^2)	**1.242**	**0.253**	**2.231**	**0.016**
U-Isop (ng/mg creat)	**6.063**	**3.257**	**8.870**	**<0.001**
R^2	0.54			
U-Isop (ng/mg creat)				
Age (Years)	−0.066	−0.160	0.028	0.159
Gender (Male)	0.274	−1.228	1.777	0.705
BMI (kg/m^2)	−0.019	−0.189	0.150	0.812
S-total 25(OH)D (ng/mL)	**−0.133**	**−0.233**	**−0.033**	**0.012**
R^2	0.38			

BMI, body mass index; P-AGT, plasma angiotensinogen; P-Ang I, plasma angiotensin I; P-Ang II, plasma angiotensin II; P-Isop, plasma isoprostanes; P-Renin, plasma renin; S-ACE, serum angiotensin-converting enzyme activity; S-total 25(OH)D, serum total 25-hydroxyvitamin D; U-AGT, urinary angiotensinogen; U-Isop, urinary isoprostanes.
Bold values: statistically significant results between groups.

modulation, principally in those patients treated with RAAS inhibitors [78]. Additionally, a positive impact of antioxidant treatment on cardiovascular morbidity and mortality was already shown in patients with end-stage renal disease, while no benefits were observed in high cardiovascular risk patients without renal disease [29,30].

A major strength of our study is that we evaluated HF patients in "real-world conditions", who, as emphasized by DeVore et al., for most health systems, represent the vast majority of patients, in opposition to the small percent enrolled in clinical trials [79]. Of note, there is a lack of unambiguous evidence of clinical efficacy for many HF guideline-directed medical therapy (GDMT) in patients not adequately represented in HF trials [80]. Moreover, in advanced HF stages, some of the recommendations with proven benefits in earlier stages of HF are no longer effective [80]. Our patients presented different HF etiologies and comorbidities, and were on polypharmacy with drugs that are known to

interfere with both RAAS and oxidative stress markers (RAAS blockers, beta-blockers, diuretics, nitrates). Although this might also be considered a limitation, we think that this study pushes forward the comprehension of the "real-world impact" on RAAS and ROS in HF patients, suggesting personalized therapeutic approaches to fulfil the still-unmet needs in HF management and prognosis.

A schematic representation of the interplay between RAAAS and oxidative stress markers in CHF patients with or without renal impairment and on optimized medical therapy is shown in Fig. 5.

5. Conclusions

CHF patients with iRF have higher intrarenal RAAS activation and lower serum vitamin D values, exhibiting several positive associations between oxidative stress and RAAS markers, in contrast to CHF patients with nRF. The inverse association of vitamin D with U-Isop, which in turn is positively associated with RAAS markers in CHF patients with renal impairment, suggests that, in these patients, severe vitamin D deficiency-driven oxidative stress limit RAAS blockers efficacy by promoting RAAS upregulation. Furthermore, our results suggest that stratifying CHF patients by renal function may be better than stratifying by functional class to identify those with intrarenal RAAS activation, which will probably benefit from the combination of RAAS blockers with vitamin D and/or antioxidants [5,28,81,82].

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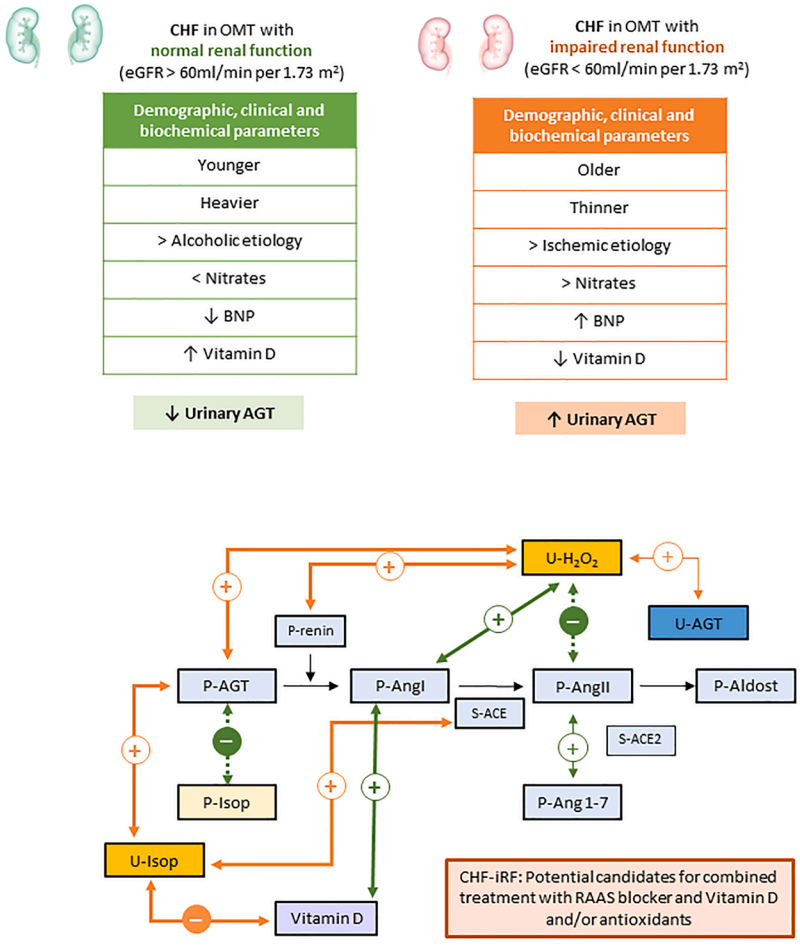

Fig. 5. Schematic representation of the interplay between RAAS and oxidative stress markers in CHF patients with normal (nRF, green) or impaired (iRF, orange) and on optimized medical therapy (OMT).
AGT, angiotensinogen; **BNP**, B-type natriuretic peptide; **CHF**, chronic heart failure; **eGFR**, estimated glomerular filtration rate; **OMT**, optimized medical therapy; **P-AGT**, plasma angiotensinogen; **P-Ang I**, plasma angiotensin I; **P-Ang II**, plasma angiotensin II; **P-Ang 1−7**, plasma angiotensin 1−7; **S-ACE**, serum angiotensin-converting enzyme; **S-ACE2**, serum angiotensin-converting enzyme type 2; **P-Isop**, plasma isoprostanes; **P-renin**, plasma renin; **RAAS**, renin-angiotensin-aldosterone system; **P-Aldost**, plasma aldosterone; **U-AGT**, urinary angiotensinogen; **U-Isop**, urinary isoprostanes; **U-H_2O_2**, urinary hydrogen peroxide.
Green filled arrows, positive correlations in CHF-nRF patients; **green dotted arrows**, negative correlations in CHF-nRF patients; **orange arrows**, positive correlations in CHF-iRF patients. **Blue boxes**, RAAS markers (light blue for plasma, dark blue for urine); **yellow boxes**, oxidative stress markers (light yellow for plasma, dark yellow for urine).

CHF-nRF: (−) **negative feedback regulation of RAAS:**
The inverse correlation between H_2O_2 and P-Ang II might be due to increased formation of P-Ang 1-7 from Ang II, since Ang 1-7 might counterregulate Ang II-induced ROS generation.
The positive correlation between H_2O_2 and Ang I might result, at least in part, from an inhibitory effect of H_2O_2 on ACE activity; in addition, H_2O_2 could also have a stimulant effect on Ang II-degrading enzymes, such as ACE2, aminopeptidases, endopeptidases, carboxipeptidases and neprilysin, which would contribute to the increase in P-Ang 1-7 levels; ROS or ROS-by products (isoprostanes) could also inhibit systemic AGT expression; Vitamin D increases Ang I probably by inhibiting ACE or other Ang II-forming enzymes.

CHF-iRF: (+) **positive feedback regulation of RAAS:**
Low Vitamin D values contribute to enhanced oxidative stress; ROS (H_2O_2) and ROS by-products (isoprostanes) increase the expression and/or activity of systemic RAAS components (AGT, renin, ACE) and intra-renal RAAS (U-AGT).

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2020.110938.

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TRABALHO 3

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ORIGINAL ARTICLE

Longer duration of obesity is associated with a reduction in urinary angiotensinogen in prepubertal children

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Abstract

Background We aimed to study the impact of obesity on urinary excretion of angiotensinogen (U-AGT) in prepubertal children, focusing on the duration of obesity and gender. Also, we aimed to evaluate whether plasma angiotensinogen (P-AGT) and hydrogen peroxide (H_2O_2) play a role in the putative association.

Methods Cross-sectional evaluation of 305 children aged 8–9 years (160 normal weight, 86 overweight, and 59 obese). Anthropometric measurements and 24-h ambulatory blood pressure monitoring were performed. Angiotensinogen (AGT) was determined by a commercial enzyme-linked immunosorbent assay (ELISA) kit and H_2O_2 by a microplate fluorometric assay.

Results U-AGT and P-AGT levels were similar across body mass index (BMI) groups and between sexes. However, boys who were overweight/obese since the age of 4 years presented lower levels of U-AGT compared with those of normal weight at the same age. In children who were overweight/obese since the age of 4, urinary H_2O_2 decreased with P-AGT.

Conclusions A higher duration of obesity was associated with decreased U-AGT in boys, thus reflecting decreased intrarenal activity of the renin–angiotensin system. Also, children with a

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longer duration of obesity showed an inverse association between urinary H_2O_2 and P-AGT. Future studies should address whether these results reflect an early compensatory mechanism to limit obesity-triggered renal dysfunction.

Keywords Angiotensinogen · Obesity · Overweight · Body mass index · Children · Hydrogen peroxide

Introduction

The prevalence of childhood obesity has tripled in the last three decades, reaching epidemic proportions around the world [1]. Even at younger ages, obesity triggers a cascade of metabolic changes that contribute to the development of hypertension, diabetes, atherosclerosis, and chronic renal disease [2, 3]. Besides, obesity in childhood and adolescence predicts the risk of cardiovascular morbidity and premature mortality in adults [4]. The mechanisms underlying the association between a high body mass index (BMI) in childhood and adolescence and cardiovascular and kidney disease in adulthood are not well characterized, and no biomarkers are available for use in routine clinical practice to allow early identification of high-risk children.

The renin–angiotensin–aldosterone system (RAAS) has been implicated in the harmful consequences of obesity. This well-coordinated hormonal system regulates cardiovascular and renal function by controlling fluid and electrolyte homeostasis. Furthermore, tissue RAAS exists in specific tissues, namely the kidney and adipose tissue [5]. In the adipose tissue, RAAS components are highly expressed and secreted by mature adipocytes [6–8], although contradictory findings have been reported regarding its regulation in obese conditions. Some studies report an increased expression [6] and secretion [5, 9] of adipose tissue-derived RAAS components in obese individuals that might contribute to activate systemic RAAS and potentiate its deleterious effects [6, 8, 10, 11]. Other studies describe reduced expression of angiotensinogen (AGT) in adipose tissue that parallels the time course of adipocyte hypertrophy, which is tightly associated with the progression of obesity [12].

Childhood obesity is also associated with renal injury, and the kidney is particularly vulnerable to the effects of the RAAS, both through the hemodynamic impact of systemic RAAS activation and by local intrarenal activation, mostly derived from AGT produced in the proximal tubule [7, 13]. Urinary excretion of AGT (U-AGT) has been validated as a noninvasive biomarker of intrarenal RAAS activity [13] and increased U-AGT has been found in human hypertension, diabetes, and renal disease [13] even in adolescents [14, 15] and children [16]. The rise in U-AGT precedes the onset of microalbuminuria, suggesting that U-AGT might be an early marker of nephropathy [15]. Interestingly, excessive activation of the adipose RAAS seems to be crucial for establishment and progression of kidney disease [13, 17] but only two small sample studies have been performed in the setting of human obesity [7, 18]. In both studies, no difference was found in U-AGT between obese adults and nonobese controls. Remarkably, it was recently highlighted that not only severity but also duration of obesity can determine obesity-related target-organ lesions [19, 20] but neither of those studies took that in consideration. Also, the study of Thethi et al. showed that obese women had higher U-AGT than obese men, although there was no gender difference in the nonobese group [7]. Gender is known to influence blood pressure (BP) and cardiovascular and renal functions, and evidence implies that the RAAS might be a putative link in this association [21]. These data raise the possibility of gender differences in the association between RAAS and obesity.

The deleterious effects of intrarenal RAAS are due, at least in part, to potentiation of angiotensin II (Ang II)-mediated renal effects [2, 7], namely the production of reactive oxygen species and cytokines and the stimulation of cell growth, inflammation and fibrosis [13, 22]. Hydrogen peroxide (H_2O_2), a nonradical oxidant and a known mediator of Ang II effects [23], also appears to be involved in the regulation of RAAS components. Recent experimental studies suggest that H_2O_2 increases AGT expression in the kidney but induces its downregulation in adipose tissue [12, 24–26]. However, this relationship between H_2O_2 and AGT has not yet been explored in humans.

We aimed at characterizing the impact of obesity on U-AGT in prepubertal, otherwise healthy, children. We compared U-AGT between normal weight, overweight, and obese children and evaluated whether gender or obesity duration influence the putative association between obesity and U-AGT at this age. Furthermore, since experimental evidence indicates H_2O_2 as a modulator of AGT expression both in kidney and adipose tissue [12, 24–26], we evaluated the link between urinary excretion of H_2O_2 (U-H_2O_2), U-AGT, and plasma AGT (P-AGT) levels.

Methods

Study design and sample collection

We conducted a cross-sectional study of children aged 8–9 years followed since birth in a previously established Portuguese cohort study (Generation XXI) [27]. Children from the original cohort ($n = 8647$ newborns) were eligible for the ObiKid project if they had anthropometric data and a blood sample withdrawn at the study site in the 7-year-old evaluation (Fig. 1; $n = 4590$). For the Obikid project [28], we wanted to include a minimum sample of 300 children, since this sample size would provide a statistical power of

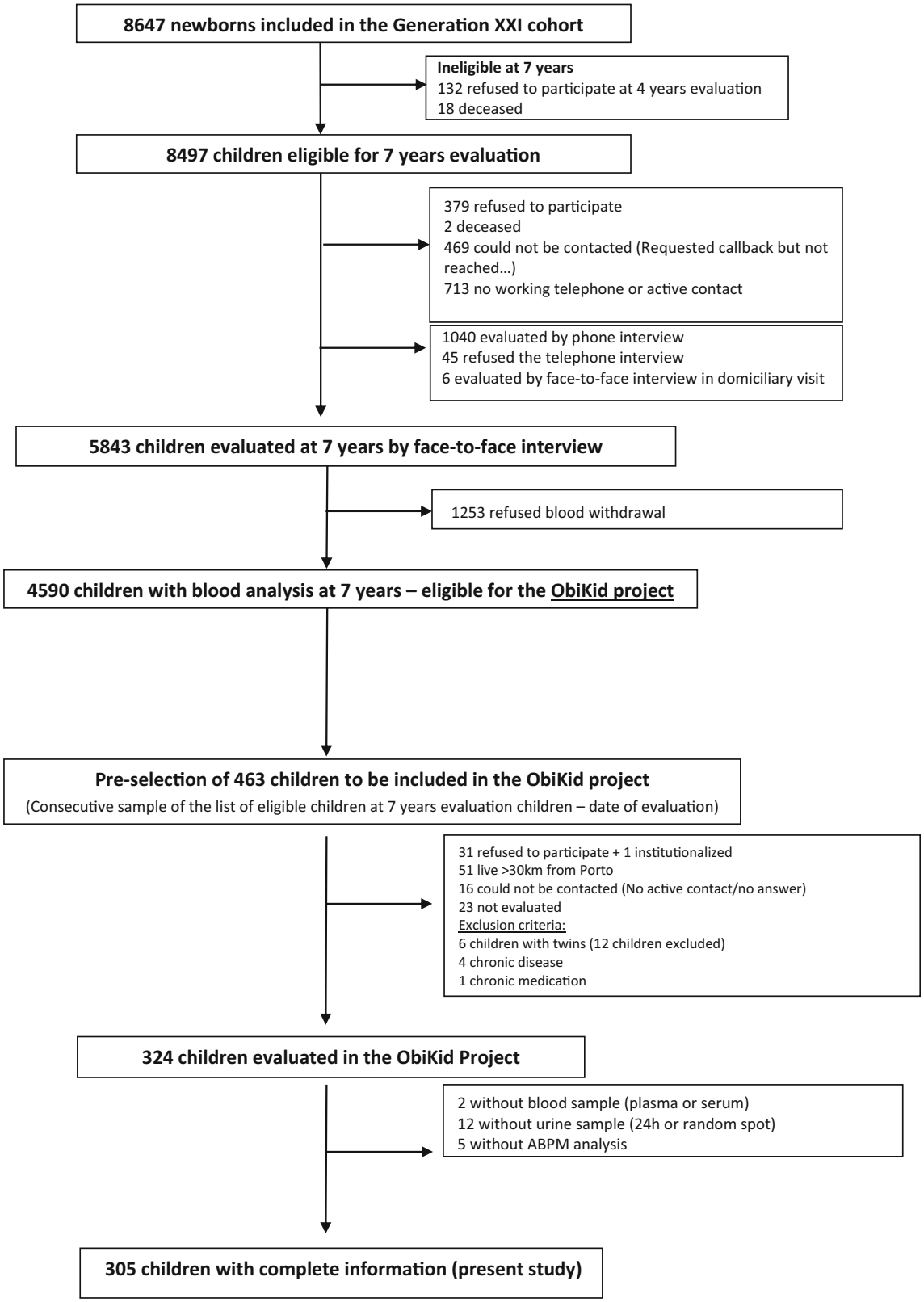

Fig. 1 Patient inclusion

85% with a significance level of 0.05 to detect a difference in estimated glomerular filtration rate (eGFR) of at least 8 ml/min/1.73 m^2 between normal weight and overweight/obese children, assuming a standard deviation (SD) of 24 and

22 ml/min/1.73 m^2 in each group, respectively [29]. We assumed that a minimum of 35% of children would be excluded due to refusal to participate, exclusion criteria, or incomplete information. We needed to consecutively screen and attempt to contact 463 children from the 4590 eligible ones to obtain the required sample; 16 could not be contacted, 32 refused to participate, 23 were unable to schedule study visits during the recruitment period, and 68 met exclusion criteria [four chronic diseases (genetic, renal, or metabolic), one chronic usage of medication (affecting BP, glucose, or lipid metabolism), 51 living far from the study site, and 12 twins]. Participants with acute illness, namely febrile conditions, were asked to postpone the scheduled study visit for a period of at least 15 days after total recovery. We finally enrolled 324 participants, but for our analysis 19 were excluded due to absence of adequate blood or urine samples or missing values in variables of interest. Our study therefore assessed 305 children (Fig. 1). No significant differences were found between included and eligible children with respect to sex, weight, height, systolic/diastolic BP, and parental education level at the follow-up visit at 7 years of age.

Data collection and variable definition

The study visits took place at the Department of Clinical Epidemiology, Predictive Medicine and Public Health, Faculty of Medicine, University of Porto. Data on birth and neonatal characteristics were abstracted from clinical records. Gestational age was considered as determined by ultrasonography. Classes of the sex-specific adequate birth weight for gestational age were defined according to the population-based Canadian reference curves (<10th percentile = small for gestational age; ≥10th percentile and <90th percentile = adequate for gestational age; ≥90th percentile = large for gestational age) [30]. Anthropometric and general physical examinations assessed weight, height, waist circumference and body composition by bioelectrical impedance analysis (percent body fat mass). Data collection was performed according to standard procedures and as previously reported [31]. Waist circumference was indexed to height [waist-to-height ratio (WHtR) in cm/m] for statistical analysis. BMI-for-age values were classified according to the World Health Organization (WHO) growth reference data for BMI z-score into the following categories: normal weight (−2 SD to +1 SD); overweight (> +1 SD), and obesity (> + 2 SD) [32]. To evaluate the effect of duration of obesity on variables studied, we gathered the most recent anthropometric data available from the previous evaluations of the cohort; accordingly, data at 4 years of age was gathered and analyzed. At that age, BMI-for-age values were classified according to the WHO growth reference data for BMI z-score for children <5 years [33]. Among overweight/obese children at the age of 8–9 with anthropometric evaluation at the age of 4 ($n = 132$), two groups were considered: those known to be overweight or obese since the age of 4 ($n = 88$; 39 girls, 49 boys) and those classified as normal weight at the age of 4 ($n = 44$; 18 girls, 26 boys).

Ambulatory BP monitoring (ABPM) was performed for 24 h with a portable, noninvasive, oscillometric BP recorder (Spacelabs Healthcare®, model 90207). The nondominant arm was used to allow free movement during everyday activity and reduce measurement errors due to movement artifacts. Cuff size was chosen according to patient's arm circumference. BP measurements were taken automatically at 20-min intervals during the day and at 30-min intervals during the night. A minimum monitoring duration of 24 h with gaps <2 h was required for acceptance; five of the ABPM analysis examinations were excluded due to insufficient readings. Readings were used to calculate mean 24 h, daytime and nighttime mean arterial (MAP), systolic (SBP) and diastolic (DBP) pressures, using SpaceLabs® software. SD scores for BP values were calculated (least mean square method) according to the published reference values of the German Working Group on Pediatric Hypertension [34]. To characterize circadian BP rhythmicity, we calculated the percentage of nocturnal fall in MAP using the following formula: [mean daytime MAP – mean nighttime MAP]/[mean daytime MAP] × 100. The nondipping pattern was considered when a drop in nighttime MAP <10% of the corresponding daytime BP was observed.

Laboratory procedures

A venous blood sample was collected from all participants after an overnight fast of at least 8 h and analyzed for cystatin C, creatinine, uric acid, aldosterone, and P-AGT. All participants collected a spot urine sample, which was analyzed for albumin, creatinine, and U-AGT, and a 24-h urine sample, which was analyzed for H_2O_2. All standard laboratory measurements were performed in the Clinical Pathology Department of Centro Hospitalar São João, Porto, Portugal. Serum cystatin C was assayed using a particle-enhanced immunonephelometric assay (N latex Cystatin C, Siemens®). Serum creatinine assay was based on the Jaffé compensated traceable to an isotope dilution mass spectrometry method (Olympus AU 5400 automated analyzer, Beckman-Coulter®, USA). Urinary creatinine and albumin were determined with the same clinical immunochemistry analyzer. GFR was estimated by the Zappitelli combined formula (in ml/min/1.73 m^2) [35]. Aldosterone concentration was measured using Liaison Aldosterone Kit® (DiaSorin Inc., USA) in serum samples. Urinary and plasma concentrations of AGT and urinary excretion of H_2O_2 were assessed in the Department of Pharmacology and Therapeutics of the Faculty of Medicine at the University of Porto. AGT was quantified by an immunoenzymatic method, using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Human Total

Angiotensinogen Assay Kit, Immuno-Biological Laboratories Co., Gunma, Japan) and H_2O_2 evaluated using a microplate fluorometric assay (Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit, Molecular Probes, Alfagene, Carcavelos, Portugal), according to protocols provided by the manufacturers. Data concerning AGT and H_2O_2 have been recently used by our group as part of studies on the same cohort to evaluate the association with urinary fibrogenic cytokines [36] and oxidative stress [37], respectively.

Statistical analysis

Data are presented as mean and SD or, if skewed, as median and 25th–75th percentiles (P25–P75). U-AGT, P-AGT, and U-H_2O_2 had an asymmetric distribution and were logarithmized (base 10) before linear regression analyzes, allowing a normal distribution to be obtained. The tertiles of WHtR, percent of body fat mass, and U-AGT were defined based on all enrolled participants. Linear trend was tested using linear regression models with BMI z-score classes and tertiles of WHtR and body fat mass included as independent continuous variables and U-AGT (logarithm base 10) as the dependent variable. Tertiles of P-AGT were used as independent variables and H_2O_2 (logarithm base 10) as the dependent variable. Models were additionally adjusted for sex and age (in months).

Results

A total of 305 children (54% male) with a mean (SD) age of 8.8 (0.2) years were assessed. General characteristics and levels of biochemical parameters are shown in Tables 1 and 2, respectively, by BMI classes (160 normal weight, 86 overweight, and 59 obese children). Overweight and obese children had higher birth weight but no differences in distribution in classes of adequacy of birth weight for gestational age. Overweight and obese children presented significantly higher values of 24-h and nighttime MAP values (Table 1). Regarding analytical parameters, overweight and obese children presented higher uric acid, creatinine, and cystatin C levels and lower eGFR values. No differences were found in median values of aldosterone, U-albumin, or U-H_2O_2 across groups (Table 2).

The levels of U–AGT were not significantly different across BMI classes (normal weight 5.6 (3.7–7.6); overweight 5.3 (3.4–8.3); obese 4.8 (2.8–7.8) μg/g of creatinine, $p = 0.338$) and neither were P–AGT levels (normal weight 36.6 (32.4–47.3); overweight 37.3 (32.6–44.8); obese 38.8 (34.1–44.3) μg/ml, $p = 0.780$) (Table 2). The distribution of U-AGT and P-AGT levels by classes of BMI and tertiles of WHtR and body fat mass is shown in Fig. 2. Regardless of the obesity index considered, no significant differences were found in the linear trend analysis of U-AGT or P-AGT levels (Fig. 2).

No differences were found between sexes in median P-AGT [38.5 (33.6–46.2) vs. 36.3 (31.7–43.4) μg/ml, $p = 0.069$, in girls and boys, respectively] or U-AGT [5.1 (2.8–8.5) vs. 5.5 (3.7–7.3) μg/g of creatinine, $p = 0.370$, in girls and boys, respectively] levels. However, in the overweight/obese group, those known to be overweight or obese since the age of 4 were more likely to excrete less U-AGT compared with those classified as normal weight at the age of 4, with this difference being significant only in boys (Table 3). This is indicated by the fact that in boys (but not in girls) who were overweight/obese at the age of 8–9 years only, a markedly higher percent of cases was found in the 2nd and 3rd tertiles of U-AGT, while in boys overweight/obese since the age of 4, the distribution of cases is relatively homogeneous across the three U-AGT tertiles (Table 3).

Linear trend analysis showed that the levels of U-H_2O_2 decreased across P-AGT tertiles in the overweight/obese group [1st tertile: 1577.0 (959.1–2008.9), 2nd tertile: 1328.9 (701.7–2380.1), 3rd tertile: 846.5 (466.0–1609.4) nmol/day, p for trend = 0.039] but not in the normal-weight group [1st tertile: 1007.5 (666.9–2030.7), 2nd tertile: 1323.4 (861.8–2133.4), 3rd tertile: 1522.2 (704.2–2055.3) nmol/day, p for trend = 0.548)] Moreover, among overweight/obese children, the effect of U-H_2O_2 on P-AGT levels was significant in those classified as overweight/obese since the age of 4, but not in those who were not (Fig. 3). The levels of U-H_2O_2 were similar across U-AGT tertiles in children who were overweight/obese at the age of 8–9; in those who were overweight/obese since the age of 4, there was a marginally significant ($p = 0.053$; Fig. 3) decrease of U-H_2O_2 values across U-AGT tertiles.

Discussion

Previous studies have shown an increase in U-AGT in children with renal disease of different etiologies [16, 38, 39], namely in adolescents with type 1 diabetes [15] or with primary hypertension [14], and in children with very low birth weight [40]. Based on these published data and on the negative impact that obesity has on the kidney [2], we would expect that overweight/obese children had higher U-AGT than age-matched controls of normal weight. However, as recently reported by our group [36], we observed no significant differences in U-AGT between normal and overweight/obese children. In this study, we further extended this result and clarified that it is observed regardless of the obesity index considered: classes of BMI or tertiles of WHtR or percent body fat mass. Although contrary to our initial hypothesis this result in obese but otherwise healthy children is in accordance with that of the only two studies concerning obesity and intrarenal RAAS,

Table 1 General characteristics of study participants by BMI z-score classes

	BMI z-score classification			*P* value
	Normal weight *n* = 160	Overweight *n* = 86	Obese *n* = 59	
General characteristics and anthropometry				
Age (months)	105.1 ± 3.0	105.2 ± 2.8	105.5 ± 2.8	0.663
Male sex	83 (52%)	43 (50%)	40 (68%)	0.069
Gestational age (weeks)	38.8 ± 1.9	38.9 ± 1.3	38.9 ± 1.2	0.891
Birth weight (g)	3185 ± 483	3266 ± 383	3354 ± 442	0.040
Birth weight for gestational age[a]				0.923
Small (<10th percentile)	22 (13.8%)	11 (12.8%)	6 (10.2%)	
Adequate (10th–90th percentile)	133 (83.1%)	72 (83.7%)	50 (84.7%)	
Large (≥90th percentile)	5 (3.1%)	3 (3.5%)	3 (5.1%)	
BMI (kg/m^2)	16.0 ± 1.2	19.5 ± 0.9	23.3 ± 2.4	<0.001
Z-score	−0.04 ± 0.74	1.56 ± 0.30	2.66 ± 0.49	<0.001
WHtR (cm/m)	44.7 ± 2.6	50.2 ± 3.2	56.6 ± 4.4	<0.001
Percent body fat mass	10.6 ± 7.1	20.0 ± 8.0	27.7 ± 9.4	<0.001
24-h ambulatory BP				
24-h MAP (mmHg)	81.1 ± 4.4	82.5 ± 5.2	82.9 ± 6.3	0.030
Z-score	0.35 ± 0.90	0.69 ± 1.07	0.51 ± 0.92	0.032
Daytime MAP (mmHg)	84.8 ± 4.6	85.8 ± 5.8	86.2 ± 6.5	0.157
Z-score	0.06 ± 0.86	0.28 ± 0.96	0.15 ± 0.96	0.219
Nighttime MAP (mmHg)	73.4 ± 4.9	74.8 ± 5.2	75.6 ± 6.3	0.011
Z-score	0.52 ± 0.91	0.85 ± 0.94	0.72 ± 0.79	0.022
MAP dipping (%)	13.4 ± 4.7	12.6 ± 5.2	12.3 ± 4.5	0.212
Nondipping pattern	34 (22%)	30 (35%)	17 (29%)	0.082

Mean ± standard deviation or *n* (%). BMI z-score classification is according to WHO criteria (normal weight, overweight, and obesity) [32]

BMI body mass index, *WHtR* waist-to-height ratio, *MAP* mean arterial pressure

[a] According to the population-based Canadian reference curves [30]

which reported similar U-AGT between obese and nonobese adults [7, 18]. In contrast, animal models of obesity show an imbalance of renal RAAS components. Obese animals have increased expression of angiotensin-converting enzyme (ACE) and of AT_1 and AT_2 receptors, but decreased expression of ACE2 and Mas receptor [41]. Adipose-derived AGT determines P-AGT concentration that, by controlling circulating AngII levels, stimulates proximal tubule production of AGT [11], thus activating intrarenal RAAS activity. Although plasma levels of AGT are increased in obese hypertensive compared with nonobese hypertensive patients [42], in our population, P-AGT was similar between normal and overweight/obese children, whatever obesity index was considered. The fact that we found similar levels of U-AGT and P-AGT might be associated with the duration of obesity (i.e., that might be not enough to have a negative impact on kidney function), activation of slow operating compensatory mechanisms, or both. Interestingly, it has been reported that obesity severity, duration, and age at onset might be crucial for the development and progression of obesity-related organ damage. Using a contemporary population from the Framingham study, Abdullah et al. suggested that a construct of obese-years should be preferable to BMI or duration of obesity alone to estimate the obesity-associated risk of type 2 diabetes [20]. Also, an adolescent onset of obesity was found to have a stronger impact on diabetes risk than adult-onset obesity [19]. When we analyzed our group of overweight/obese children according to the duration of overweight/obesity, we observed those with a longer duration presented lower levels of U-AGT than those with recent offset of overweight/obesity. This was quite unexpected given that if renal RAAS progressively contributes to renal damage, along with the duration of excessive weight, then children already overweight/obese by the age of 4 would show higher U-AGT. Some children in our population were identified as being obese at the age of 4 but as being of normal weight at the age of 8–9; however, this group was too small to be analyzed.

Interestingly, we previously reported [28] an already lower eGFR in these overweight/obese children, although renal function was in the normal range for all children. Also, we

Table 2 Laboratory data by BMI z-score classes

	BMI z-score classification			*P* values
	Normal weight *n* = 160	Overweight *n* = 86	Obese *n* = 59	
Blood analytical parameters				
Uric acid (mg/dl)	3.5 ± 0.7	3.8 ± 0.7	4.0 ± 0.8	<0.001
Creatinine (mg/dl)	0.43 ± 0.06	0.45 ± 0.06	0.45 ± 0.06	0.003
Cystatin-C (mg/L)	0.64 ± 0.07	0.67 ± 0.08	0.68 ± 0.06	<0.001
eGFR (ml/min/1.73 m^2)	138.4 ± 15.8	132.2 ± 16.4	132.1 ± 13.2	0.003
Aldosterone (ng/dl)	9.4 (7.1–13.2)	12.1 (7.5–16.2)	9.6 (8.4–13.8)	0.216
P-AGT (μg/ml)[a]	36.6 (32.4–47.3)	37.3 (32.6–44.8)	38.8 (34.1–44.3)	0.780
Urinary analytical parameters				
U-albumin (mg/g creatinine)	4.1 (2.6–7.6)	3.6 (2.1–7.8)	3.7 (2.3–5.6)	0.560
U-AGT (μg/g creatinine)[a]	5.6 (3.7–7.6)	5.3 (3.4–8.3)	4.8 (2.8–7.8)	0.338
U-H_2O_2 (nmol/day)[b]	1282.0 (711.1–2058.7)	1328.9 (680.5–1988.5)	1276.2 (701.7–2187.2)	0.855

Mean ± standard deviation or median (P25–P75). BMI z-score classification is according to WHO criteria (normal weight, overweight, and obesity) [32]

BMI body mass index, *eGFR* estimated glomerular filtration rate by Zappitelli combined formula, *P-AGT* plasma angiotensinogen, *U-albumin* urinary albumin-to-creatinine ratio, *U-AGT* urinary angiotensinogen, *U-H_2O_2* urinary hydrogen peroxide

Data concerning AGT ([a]) and H_2O_2 ([b]) have been recently used by our group as part of studies on the same cohort intended to evaluate the association with urinary fibrogenic cytokines [36] and oxidative stress [37], respectively

recently reported [36] that urinary excretion of the fibrogenic cytokines endothelin-1 (ET-1) and transforming growth factor-β1 (TGF-β1) was not increased in obese children in our cohort; indeed, they showed lower levels of urinary ET-1 and TGF-β1 than those of normal weight. Furthermore, U-AGT was associated with urinary excretion of ET-1 and TGF-β1 [23]. Of note, although there are reports of human studies showing increased expression of AGT messenger RNA (mRNA) in subcutaneous adipose tissue of obese individuals [43], most human and animal studies report a decrease [9, 12, 44]. Moreover, expression and secretion of AGT decreases as adipocytes become hypertrophied. This effect seems to be, at least in part, mediated by reactive oxygen species, namely, H_2O_2 [12]. Consistent with this, we observed an inverse association between P-AGT and U-H_2O_2 in overweight/obese children but not in normal-weight controls. Moreover, that association was seen only in children overweight/obese by the age of 4. So, although further studies are needed to fully evaluate and characterize this point, our results suggest that childhood obesity is associated with time-dependent alterations in intrarenal RAAS activity (even when established markers of renal function are in the normal range) and with an association between P-AGT and U-H_2O_2.

It is noteworthy that the difference observed in U-AGT according to duration of obesity was only significant in boys. In girls, the levels of U-AGT were not associated with the duration of overweight/obesity . Studies in animal models suggest that the kidney has estrogen receptors that can mediate protective effects against renal injury by attenuating glomerulosclerosis and tubulointerstitial fibrosis [45, 46]. Interestingly, this protection has been suggested to involve both the RAAS and the endothelin system [45]. The children in our study are at a prepubertal age and we were not able to quantify neither Tanner stages nor sex hormones. As such, although we recruited a homogeneous population, we cannot conclude whether prepubertal girls already have higher levels of estrogens and androgen metabolites than prepubertal boys, as reported elsewhere [47, 48]. Moreover, the fact that obesity might anticipate puberty in girls and delay it in boys [49] could somehow blunt the impact of obesity on U-AGT levels. In other words, the absence of difference in U-AGT according to duration of obesity in girls could also be explained by the fact that girls in the obese group were at a more advanced phase of pubertal development. In line with this, we also observed that boys excreted significantly more H_2O_2 than girls (unpublished observations) and that in boys, higher salt consumption was associated with higher systolic BP, specifically in those overweight/obese [50].

Major strengths of our study are inclusion of a large and homogeneous sample of healthy prepubertal children and the gathering of data on 24-h ABPM and renal function from all of them. The cross-sectional design of our study might be seen as a limitation. However, as our cohort will be followed until adulthood, we expect to re-evaluate these children in the next evaluation period, when they will be adolescents, and it will

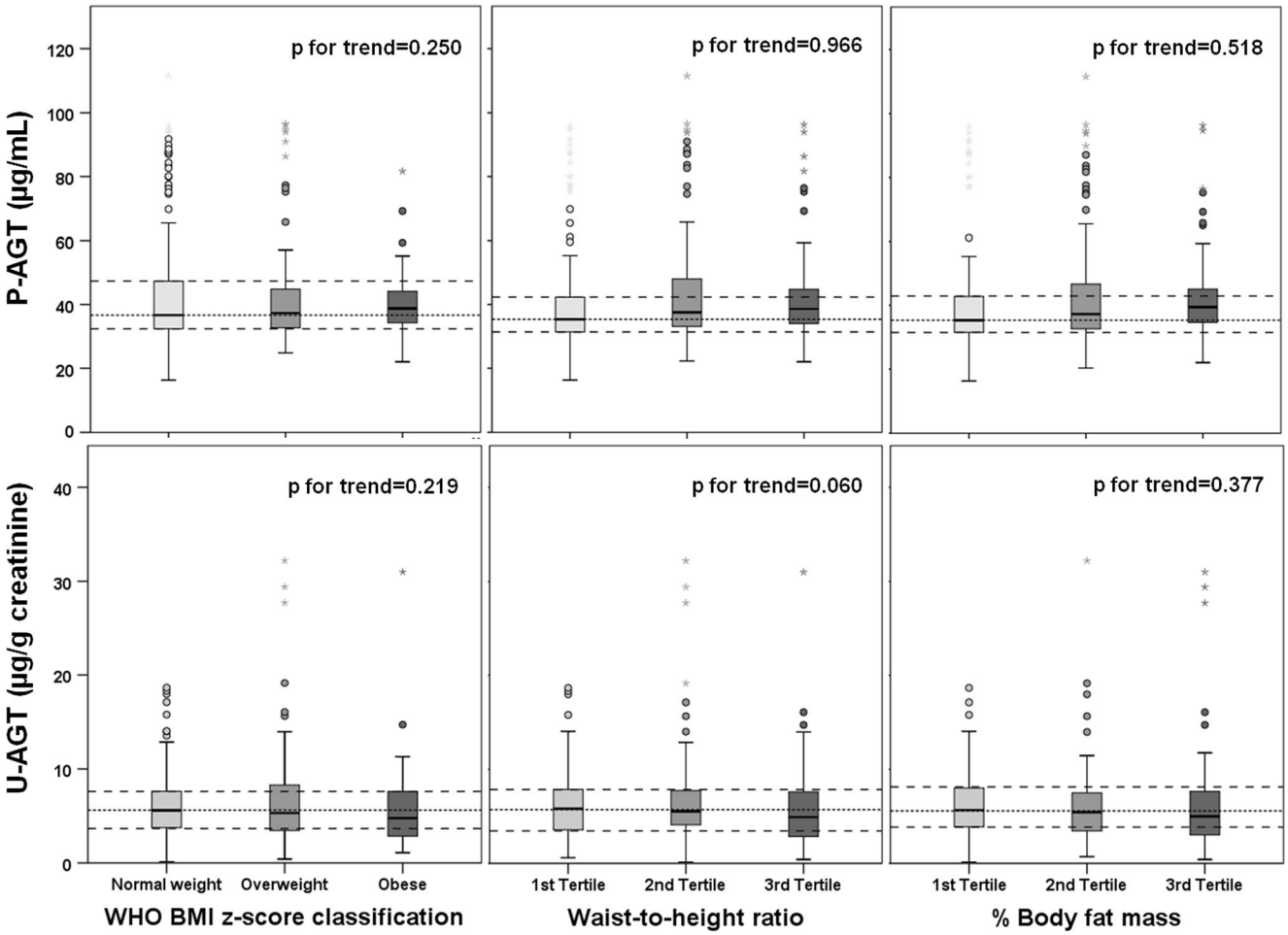

Fig. 2 Distribution of P-AGT and U-AGT by classes of BMI and tertiles of waist-to-height ratio and percent body fat mass. Normal weight, overweight, and obesity group classification is according to the WHO classification for BMI z-score [32]. Tertiles of waist-to-height ratio (≤45.65; 45.66–50.00; >50.00) and percent of body fat mass (≤11.10; 11.11–20.70; >20.70) were divided based on all enrolled participants. P-AGT and U-AGT data is expressed as medians and percentiles 25 and 75. *P* values for linear trend across groups were calculated by linear regression using U-AGT as dependent variable (logarithm base 10) and adjusting for sex and age in months. *Horizontal dashed lines* represent the 25th, 50th, and 75th percentiles in the normal-weight group for each parameter. *WHO* World Health Organization, *BMI* body mass index, *P-AGT* plasma angiotensinogen, *U-AGT* urinary angiotensinogen

be possible to perform a longitudinal analysis to complement and expand the hypothesis raised in this study. In addition, longitudinal studies should include a group of children that has experienced weight loss to test whether that would protect them from metabolic changes later in life.

Our results reinforce the importance of encouraging preventive measures targeting obesity even in prepubertal children, since obesity seems to have an impact on kidney function even at this young age. Moreover, data on sex hormones and Tanner stages would have enriched our analysis significantly.

Table 3 Distribution of cases (%) across urinary excretion of angiotensinogen (U-AGT) tertiles

	U-AGT (µg/g creatinine)		
	1st tertile	2nd tertile	3rd tertile
Boys ($p = 0.036$)			
Overweight/obese since the age of 4	36.7%	34.7%	28.6%
Overweight/obese only at the age of 8–9	11.5%	61.5%	26.9%
Girls ($p = 0.383$)			
Overweight/obese since the age of 4	48.7%	12.8%	38.5%
Overweight/obese only at the age of 8–9	38.9%	27.8%	33.3%

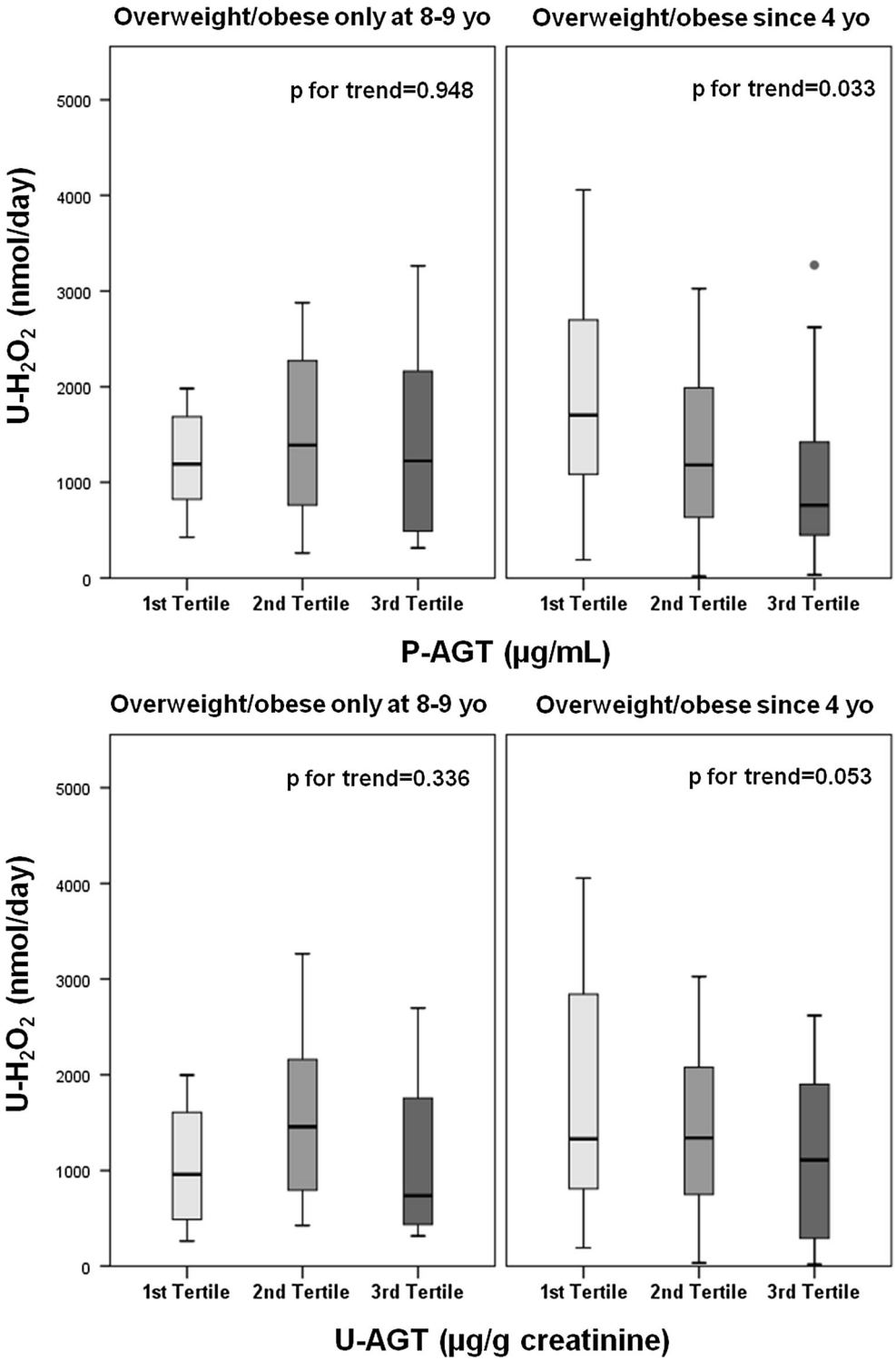

Fig. 3 Distribution of U-H_2O_2 by tertiles of P-AGT and U-AGT, according to duration of overweight/obesity. Tertiles of P-AGT (≤35.0; 35.0–41.5; >41.5) and U-AGT (≤4.1; 4.1–6.7; >6.7) were divided based on all enrolled participants. U-H_2O_2 data is expressed as medians and percentiles 25 and 75. *P* values for linear trend across groups were calculated by linear regression using U-H_2O_2 as dependent variable (logarithm base 10) and adjusting for sex and age in months. *WHO* World Health Organization, *BMI* body mass index, *U-H_2O_2* urinary hydrogen peroxide, *P-AGT* plasma angiotensinogen, *U-AGT* urinary angiotensinogen

Alternatively, studies in prepubertal children should be conducted at even youger ages so that the differences in hormonal levels would not bias the interpretation of the associated results. Certainly, future analysis by our group will pursue this issue.

In conclusion, although intrarenal RAAS activity in overweight and obese children was similar to that of a normal-weight control group, overweight/obese boys —but not girls— with a longer duration of obesity presented lower levels of U-AGT and an inverse association between U-H_2O_2 and P-AGT. This might tempt one to think that ACE inhibitors or angiotensin receptor bolckers (ARBs) could be less effective in protecting the kidney of obese patients. However, this study is

the first to address the role of the RAAS in the context of obesity-induced renal damage. Also, it focused on short-lasting, early childhood obesity, which might not have the same consequences as chronic obesity. Therefore, further studies are essential (including longitudinal studies) to support evidenced-based guidelines for obese patients, since this population has only been addressed in expert opinion and not in actual guidelines.

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Compliance with ethical standards The Generation XXI study was approved by the Ethics Committee of Centro Hospitalar São João, E.P.E. and Faculty of Medicine of the University of Porto, Portugal, and by the National Data Protection Commission. It complies with the Helsinki Declaration and the current national legislation. Written informed consent from parents (or their legal substitute) and verbal assent from children was obtained, concerning information and biological samples gathering.

Conflicts of interest None of the authors have any financial or nonfinancial competing interests concerning the present study.

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TRABALHO 4

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Interaction between the Renin–Angiotensin System and Enteric Neurotransmission Contributes to Colonic Dysmotility in the TNBS-Induced Model of Colitis

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Abstract: Angiotensin II (Ang II) regulates colon contraction, acting not only directly on smooth muscle but also indirectly, interfering with myenteric neuromodulation mediated by the activation of AT_1 /AT_2 receptors. In this article, we aimed to explore which mediators and cells were involved in Ang II-mediated colonic contraction in the TNBS-induced rat model of colitis. The contractile responses to Ang II were evaluated in distinct regions of the colon of control animals or animals with colitis in the absence and presence of different antagonists/inhibitors. Endogenous levels of Ang II in the colon were assessed by ELISA and the number of AT_1/AT_2 receptors by qPCR. Ang II caused AT_1 receptor-mediated colonic contraction that was markedly decreased along the colons of TNBS-induced rats, consistent with reduced AT_1 mRNA expression. However, the effect mediated by Ang II is much more intricate, involving (in addition to smooth muscle cells and nerve terminals) ICC and EGC, which communicate by releasing ACh and NO in a complex mechanism that changes colitis, unveiling new therapeutic targets.

Keywords: inflammatory bowel disease; IBD; TNBS-induced colitis; colonic dysmotility; angiotensin II; AT_1 and AT_2 receptors; nitric oxide; interstitial cells of Cajal; ICC; enteric glial cells; EGC

1. Introduction

Inflammatory bowel disease (IBD) includes Crohn's disease (CD) and ulcerative colitis (UC). It is a chronic inflammatory condition of the gastrointestinal (GI) tract with prevalence surpassing 0.3% in western countries [1], and it negatively influences the health status and quality of life of patients [2,3], in part due to alterations in colonic transit and reactivity such as the urge to defecate and/or bloody diarrhea [2,3]. Studies on the etiopathology of IBD have mainly focused on immune mechanisms [4,5], and current therapeutic approaches are not always effective in inducing and/or maintaining remission, often depending on type, localization and severity of the disease [6]. Therefore, other pathophysiological mechanisms of IBD should be explored in order to identify new drug targets to improve treatment.

It is nowadays clearly accepted that the renin–angiotensin system (RAS) is involved in inflammation [7], including that of the GI tract [8–11]. IBD patients who were on angiotensin II (Ang II) type I (AT_1) receptor antagonists showed less expression of pro-inflammatory cytokines in colonic biopsies than IBD patients who were not on these drugs [8]. Moreover, patients with active CD presented higher levels of Ang II in the intestinal mucosa than healthy controls or patients with UC [12]. Ang II is the main effector peptide of the system and triggers its functions via AT_1 and AT_2 receptors [13]. AT_1 receptors mediate all classic actions of Ang II such as contraction of smooth muscle cells, inflammation, fibrosis, cellular growth and release of aldosterone, while activation of AT_2 receptors appears to counterbalance those effects [14]. Particularly in the colon, both AT_1 and AT_2 receptors were found in the surface epithelium, even though AT_2 receptors presented a weaker expression [12]. In rats, Ang II induces contractile responses in intestinal muscles via activation of neural AT_1 receptors and/or AT_1 receptors located postjunctionally in smooth muscle cells [12,14–16]. Although AT_2 receptors are described in the GI tract, their role in intestinal contraction is only now starting to be reported [17]. The contraction caused by Ang II in the distal colon is lower in experimental IBD than in controls, apparently through recruitment of AT_2 receptors [17]. Interestingly, it was suggested that enteric neurons participate in Ang II-mediated colonic contraction in dextran sulfate sodium (DSS)-induced colitis in rats [17].

The pathophysiology of IBD is complex in part due to the complex gut network that engages smooth muscle cells, epithelial cells and the intricate enteric nervous system (ENS) to promote gut motility and nutrient absorption [18]. The ENS comprises complex networks of enteric neurons that interact with enteric glial cells (EGC) and interstitial cells of Cajal (ICC) [18,19]. Recently, an involvement of EGC in controlling GI functions has been suggested [18], namely gut motility and secretions [20,21], but ECG are also capable of responding to injury, stress and inflammation [22], producing both pro- and anti-inflammatory responses [23]. In fact, in CD patients [22] and in TNBS-induced ileitis in the guinea pig [24], glial-mediated reactions and EGC mitosis, respectively, were found to have increased. Additionally, ICC are described as the pacemaker cells of the GI tract, regulating spontaneous contractions of the gut [25] and gut motility [26]. In fact, several studies point to a role for ICC in mediating nitrergic [25,27] and purinergic [28] relaxation as well as cholinergic contraction [26] of the GI smooth muscle. In IBD, lower levels of ICC [29] as well as structural changes in these cells have been found [30]. There is almost no information on a putative role of these non-neuronal cells in Ang II-mediated responses, namely colonic contraction.

The aim of our study was to characterize the contractile response to Ang II in three regions of the colon—proximal colon (PC), middle colon (MC) and distal colon (DC)—of rats with 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced IBD [31] and to look for which cells and mediators participate in that effect of Ang II.

2. Results

2.1. Assessment of Colitis

The body weight and number of pellets were quantified along the time of induction of colitis. From day 1 on, TNBS-induced rats presented lower body weight change than controls (Figure 1A), with a total body weight loss of 5% on day 7. The number of fecal pellets of TNBS-induced animals was also lower than that of controls; this difference was particularly evident at the beginning, but on day 3, the number of fecal pellets from TNBS-induced rats started to increase and was similar to that of controls by the end of the protocol (Figure 1B).

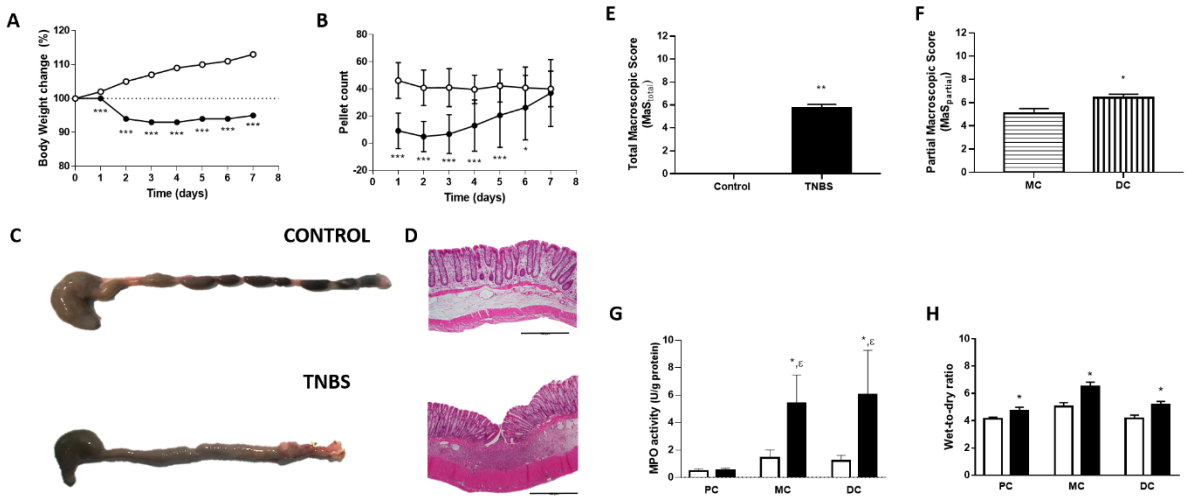

Figure 1. (**A**) Body weight change of control (white circles, n = 28) and TNBS-induced rats (black circles, n = 87). (**B**) Number of fecal pellets of control (white circles, n = 28) and TNBS-induced rats (black circles, n = 87). (**C**) Macroscopic and (**D**) microscopic images from the colons of control and TNBS-induced animals. (**E**) Macroscopic score (MaS) of control (white bar, n = 28) and TNBS-induced rats (black bar, n = 87). (**F**) MaS of the middle colon (MC) and the correspondent distal colon (DC) of TNBS-induced rats (n = 87). (**G**) Myeloperoxidase (MPO) activity in the proximal colon (PC), middle colon (MC) and distal colon (DC) of control (white bars; PC: n = 12, MD: n = 9, DC: n = 9) and TNBS-induced rats (black bars; PC: n = 16, MD: n = 11, DC: n = 8). (**H**) Wet-to-dry ratio in the proximal colon (PC), middle colon (MC) and distal colon (DC) of control (white bars; PC: n = 29, MC: n = 21, DC: n = 21) and TNBS-induced rats (black bars; PC: n = 20, MC: n = 17, DC: n = 19). * $p < 0.05$ vs. corresponding controls (except in (D), where * $p < 0.05$ vs. MC), ** $p < 0.0001$, *** $p < 0.000001$ vs. control animals and $^{\varepsilon}$ $p < 0.05$ vs. TNBS-PC.

Upon macroscopic evaluation, it was evident that the colons of TNBS-induced rats were filled with pasty stools in their entire lengths and appeared to have a larger diameter than those of control animals, which were filled with ovoid-shaped fecal pellets (Figure 1C). TNBS-induced rats presented a thicker mucosa that usually showed areas of ulceration and hyperemia, particularly in the DC (Figure 1C). Microscopic evaluation of samples from the colon of TNBS-induced rats showed thickening of the colon wall, with several erosive/ulcerative lesions, moderate to extensive inflammatory infiltrate in the mucosa and sub-mucosa and occasional areas of re-epithelization and fibrosis (Figure 1D). TNBS-induced rats had a higher MaS than control animals (Figure 1E). Of interest, in TNBS-induced rats, partial MaS of the DC was higher than that of the MC (Figure 1F).

Colonic inflammation was also confirmed by increased MPO activity in the MC and DC of TNBS-induced animals when compared to the correspondent colonic segment of controls and compared to that of the PC of TNBS-induced animals (Figure 1G). No differences were found between the PC of control and TNBS-induced rats (Figure 1G). In addition, the wet-to-dry ratio (a marker of edema) was higher in all colonic portions of TNBS-induced animals compared with those of control animals (Figure 1H).

2.2. Colonic Portions Reactivity to Angiotensin II and Peptide Levels

Exogenous administration of Ang II caused a concentration-dependent contraction of the PC, MC and DC in both control and TNBS-induced rats (Figure 2). Interestingly, the contraction promoted by exogenous Ang II was different depending on the colonic region observed and on the units in which data was analyzed.

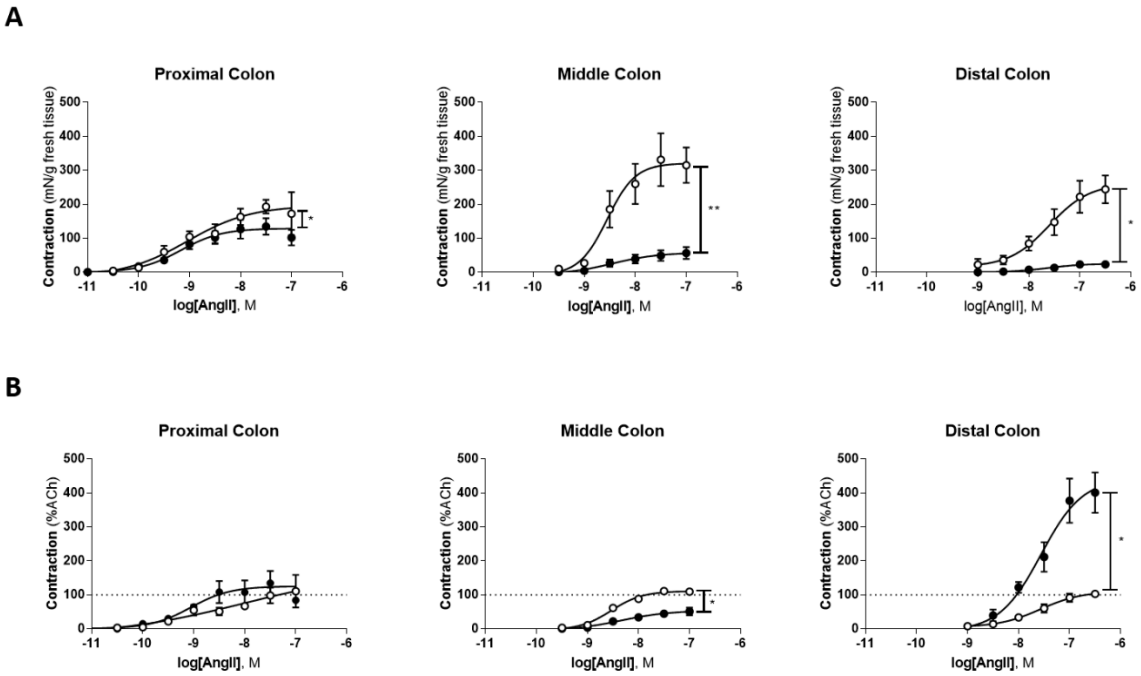

Figure 2. Concentration-response curves to Ang II. (**A**) Ang II-mediated response expressed as tension (mN/g of fresh tissue) in the proximal colon (controls, open circles, n = 7 vs. TNBS-induced rats, black circles, n = 10), middle colon (controls, open circles, n = 5 vs. TNBS-induced rats, black circles, n = 10) and distal colon (controls, open circles, n = 6 vs. TNBS-induced rats, black circles, n = 6); (**B**) Ang II-mediated response expressed as a percentage of ACh (%ACh) in the proximal colon (controls, open circles, n = 7 vs. TNBS-induced rats, black circles, n = 9), middle colon (controls, open circles, n = 5 vs. TNBS-induced rats, black circles, n = 10) and distal colon (controls, open circles, n = 6 vs. TNBS-induced rats, black circles, n = 6). Vertical lines point to statistically significant differences in the E_{max} of the corresponding groups, * $p < 0.05$, ** $p < 0.0001$.

First, we expressed the contractile response to Ang II as mN of tension *per* g of fresh tissue to have information on the capacity of Ang II to contract a segment of the colon (control and TNBS-induced group). The contraction (mN/g) induced by Ang II was markedly lower in all three colonic portions of TNBS-induced rats than in the correspondent colonic portions of the control group (Figure 2A) (Table 1). Next, we expressed the contractile response to Ang II as a percentage of that induced by ACh 10 µM in the same tissue (%ACh) in order to gather information on the relative capacity of Ang II to contract a segment of the colon when compared to that of another contracturant in the same tissue sample; we selected ACh as the classical contracturant of the colon (ACh). In this case, the contractile response (%ACh) induced by Ang II differed along the colon (Figure 2B). In the PC, there was no difference between control and TNBS-induced animals (Figure 2B) (Table 1), in the MC the response to Ang II was lower in TNBS-induced animals than in controls (Figure 2B) (Table 1) and in the DC Ang II-mediated contraction was markedly higher in TNBS-induced animals than in controls (Figure 2B) (Table 1). Together, these results show that the contractile response to Ang II is decreased in TNBS-induced rats when compared to control rats, but this decrease is not the consequence of a generalized decreased contractile capacity of the inflamed colon. In the PC, that decrease is accompanied by a proportional decrease in the response to Ach, but in the MC, the decrease in Ang II-induced contraction is more marked than that of ACh, while in the DC, the contractile capacity of Ang II is markedly higher than that of ACh (even though Ang II has less force of contraction (mN/g) in TNBS-induced than in control rats). There was no difference in the EC_{50} between groups for the same region of the colon ($p > 0.05$, data not shown).

Table 1. E_{max} (mN/g and %ACh) for Ang II-mediated contraction in the PC, MC and DC of control and TNBS-induced rats.

	PC	MC	DC
	Emax (mN/g)	**Emax (mN/g)**	**Emax (mN/g)**
Control	199.7 ± 27.38	334.3 ± 61.72	329.8 ± 51.59
TNBS	103.9 ± 25.05 *	43.76 ± 12.86 **	27.12 ± 7.295 *
	Emax (% ACh)	**Emax (% ACh)**	**Emax (% ACh)**
Control	100.2 ± 21.42	115.2 ± 5.009	142.1 ± 18.98
TNBS	102.8 ± 29.95	43.11 ± 9.518 *	467.3 ± 80.05 *

* $p < 0.05$ vs. corresponding CTRL animals; ** $p < 0.0001$ vs. corresponding CTRL animals.

The quantification of the endogenous levels of Ang II in the colonic tissue (PC, MC and DC) of control and TNBS-induced rats showed higher levels of Ang II in the PC and the DC of TNBS-induced animals than in control animals but similar levels between experimental groups in the MC ($p > 0.05$) (Figure 3). Intriguingly, in control rats, the endogenous colonic levels of Ang II were higher in the MC than in the PC and DC (Figure 3). No statistical differences were found between the levels of endogenous Ang II among the colonic portions of TNBS-induced rats (Figure 3).

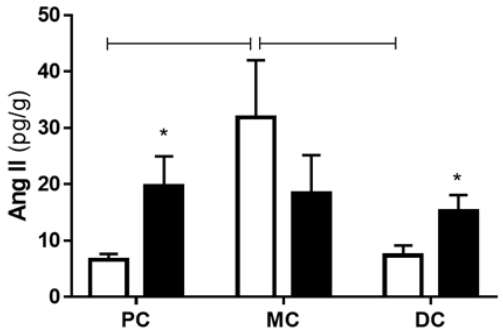

Figure 3. Levels of Ang II of control (white bars) and TNBS-induced rats (black bars) in the PC (controls: $n = 5$; TNBS: $n = 4$), the MC (controls: $n = 5$; TNBS: $n = 5$) and the DC (controls: $n = 6$; TNBS: $n = 5$). * $p < 0.05$ vs. corresponding control; capped lines point to statistically significant differences between groups with $p < 0.05$.

2.3. Role and Expression of Angiotensin II Type I and Type II Receptors

To further characterize the colonic contractile effect induced by Ang II, the role of AT_1 and AT_2 receptors was assessed. Candesartan 10 nM, an AT_1 receptor antagonist, abolished the contractile effect mediated by Ang II in all three colonic regions of control animals (Figure 4A) and of the TNBS-induced group (Figure 4B), indicating that this was the receptor responsible for the Ang II-mediated contraction. Differently, the presence of the AT_2 receptor antagonist PD123319 100 nM increased the contractile response to Ang II in the PC, MC and DC of control animals (Figure 4C) but had no effect on the contractile response to Ang II in TNBS-induced rats ($p > 0.05$) (Figure 4D). These results indicate that in control rats, AT_2 receptors also mediate Ang II-induced contraction but have a contrary effect to AT_1 receptors (promoting relaxation). In addition, these results show that this AT_2 receptor-mediated regulation is absent in TNBS-induced rats.

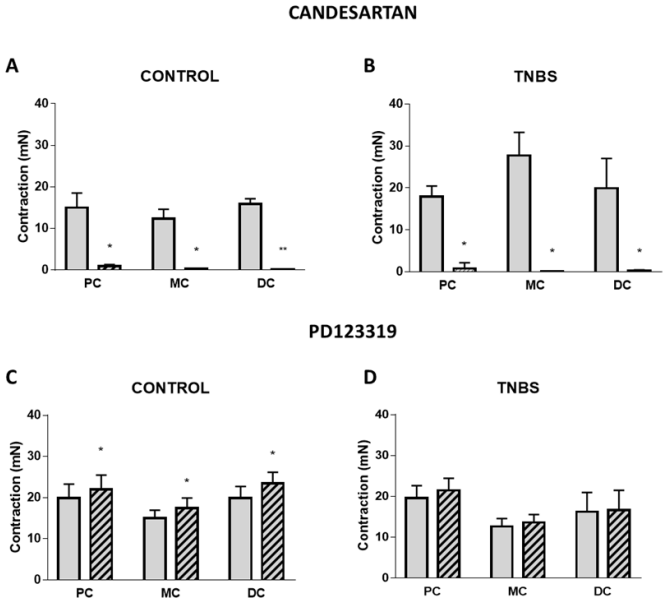

Figure 4. Characterization of the receptors involved in the contraction induced by Ang II in PC, MC and DC. Gray bars and striped bars represent, respectively, the absence and presence of Candesartan 10 nM in (**A**) control (PC: $n = 6$; MC: $n = 6$; DC: $n = 6$) and (**B**) TNBS-induced animals (PC: $n = 6$; MC: $n = 7$; DC: $n = 7$) and the absence and presence of PD123319 100 nM in (**C**) control (PC: $n = 7$; MC: $n = 6$; DC: $n = 7$) and (**D**) TNBS-induced animals (PC: $n = 11$; MC: $n = 9$; DC: $n = 8$). * $p < 0.05$ and ** $p < 0.0001$ vs. Ang II alone.

The expression of the AT_1 receptor appeared to be decreased in TNBS-induced animals, even though a statistically significant difference was only found in the PC (Figure 5A). Differently, the expression of the AT_2 receptor was not statistically altered in TNBS-induced rats, although the average values of expression of the AT_2 receptor in the DC of TNBS-induced rats were numerically higher (Figure 5B). Overall, these results point to decreased expression of the AT_1 receptor but similar expression of the AT_2 receptor in the colons of TNBS-induced rats when compared with controls.

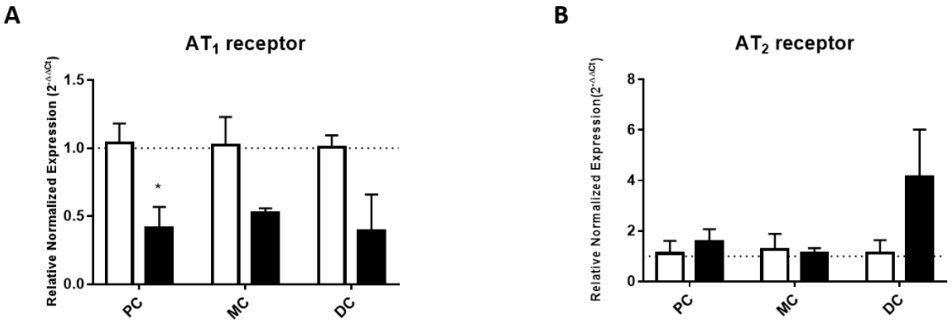

Figure 5. Relative normalized expression ($2^{-\Delta\Delta C}{}_t$) of (**A**) AT_1 (PC: $n = 3$, MC: $n = 2$; DC: $n = 3$) and (**B**) AT_2 (PC: $n = 2$; MC: $n = 3$; DC: $n = 2$) receptors in the controls (white bars) and TNBS-induced (black bars) animals. * $p < 0.05$ vs. corresponding control group.

2.4. Neural and Non-Neural Cells Mediating Angiotensin II-Induced Colonic Contraction

Because the colonic tissue is a complex network involving different types of cells, we further evaluated the response to different antagonists and blockers. Tetrodotoxin (TTX 1 μM), a neurotoxin that blocks Na^+ voltage-gated ion channels impairing neurotransmitter release from myenteric neurons, significantly increased the contractile response to Ang II in all colonic regions of both control (Figure 6A left) and TNBS-induced rats (Figure 6A right).

Further analysis revealed a statistically significant difference between control and TNBS-induced groups ($p < 0.05$) but not between tissues nor an interaction between the two factors ($p > 0.05$).

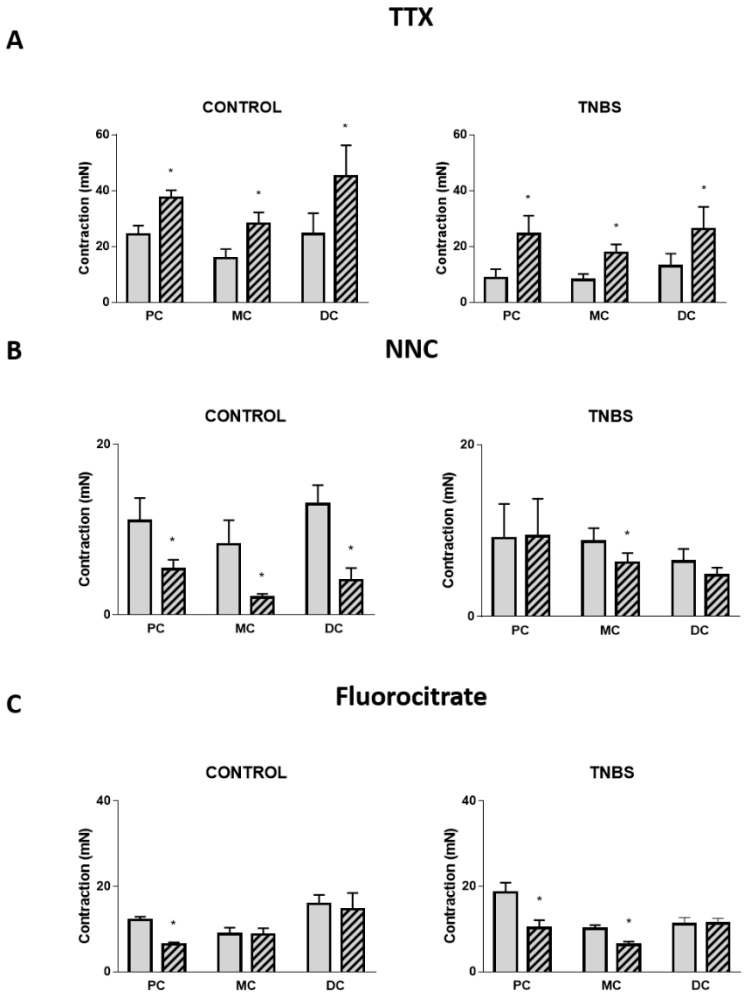

Figure 6. Contractile response to Ang II in the PC, MC and DC in the absence (gray bars) and presence (striped bars) of (**A**) TTX 1 μM in control (PC: $n = 6$; MC: $n = 5$; DC: $n = 5$) and TNBS-induced animals (PC: $n = 6$; MC: $n = 4$; DC: $n = 6$); (**B**) NNC 55-0396 10 μM (PC) or 30 μM (MC and DC) in control (PC: $n = 8$; MC: $n = 8$; DC: $n = 6$) and TNBS-induced animals (PC: $n = 6$; MC: $n = 5$; DC: $n = 6$); and (**C**) fluorocitrate 300 μM in control (PC: $n = 7$; MC: $n = 5$; DC: $n = 5$) and TNBS-induced animals (PC: $n = 5$; MC: $n = 6$; DC: $n = 7$). * $p < 0.05$ vs. Ang II alone.

NNC 55-0396 (a calcium T-type channel blocker, 10 μM or 30 μM) was used as an ICC inhibitor. This drug decreased Ang II-induced contraction in all colonic portions of control animals (Figure 6B left). However, in TNBS-induced animals, this effect was limited to the MC (Figure 6B right). Further analysis revealed a statistically significant difference between control and TNBS-induced groups ($p < 0.05$) but not between tissues nor an interaction between the two factors ($p > 0.05$).

Furthermore, in control animals, fluorocitrate (FC, 300 μM), a glial cells' inhibitor, decreased the contractile response to Ang II in the PC but had no effect in the other portions of the colon (Figure 6C left). In TNBS-induced animals, this effect was visible not only in the PC, but also in the MC (Figure 6C right). Further analysis revealed a statistically significant difference between colonic regions ($p < 0.05$) but not between groups nor an interaction between the two factors ($p > 0.05$).

Overall, these results indicate that Ang II-induced colonic contraction is under the regulation of both neuronal (inhibitory) and non-neuronal cells (excitatory), and that the non-neuronal component is altered in TNBS-induced colitis.

2.5. Mediators Involved in Angiotensin II-Induced Colonic Contraction

We also assessed the role of several mediators in Ang II-mediated contraction of the colon. A nitric oxide synthase (NOS) inhibitor (L-NAME, 100μM) increased the contractile response to Ang II in the PC and MC of control rats (Figure 7A left) and in the PC and DC of TNBS-induced rats (Figure 7A right). Atropine, a non-selective muscarinic antagonist, increased the contractile response to Ang II in the PC and MC of control rats (Figure 7B left) and in the PC of TNBS-induced rats (Figure 7B right). Phentolamine increased the contractile response to Ang II in the DC of control rats (17.86 ± 8.40 mN vs. 20.38 ± 8.50 mN, Ang II alone vs. Ang II + phentolamine, respectively; $p < 0.05$, n = 5; graphs not shown). Propranolol, caffeine or suramine did not alter the response to Ang II, either in control or TNBS-induced rats, in any of the three colonic portions analyzed ($p > 0.05$; data not shown).

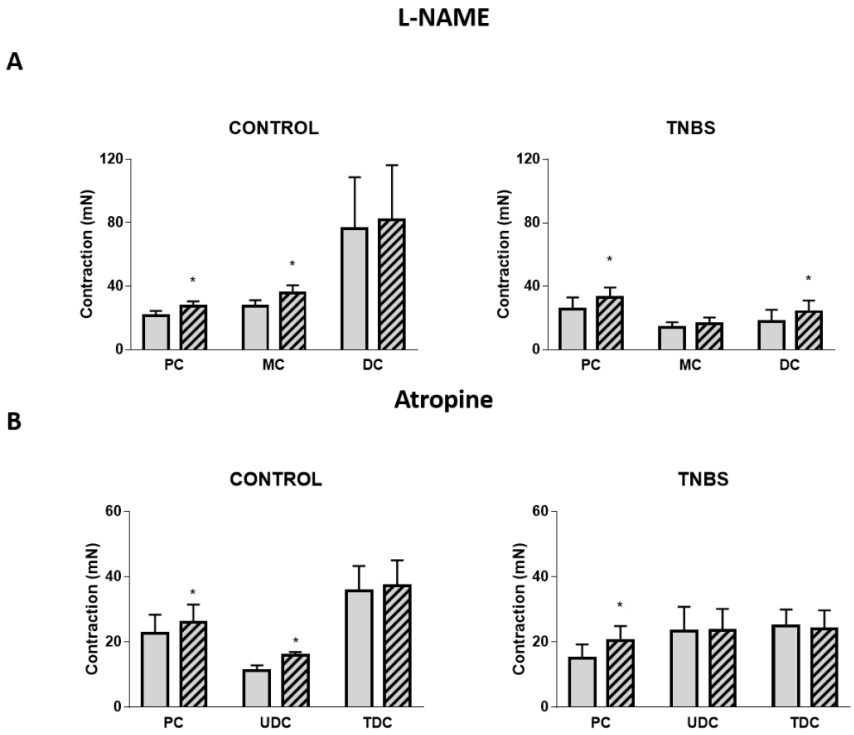

Figure 7. Contractile response to Ang II in the PC, MC and DC in the absence (gray bars) and presence (striped bars) of (**A**) L-NAME 100 μM in control (PC: $n = 5$; MC: $n = 6$; DC: $n = 6$) and TNBS-induced animals (PC: $n = 6$; MC: $n = 5$; DC: $n = 6$); and (**B**) atropine 1 μM in control (PC: $n = 5$; MC: $n = 6$; DC: $n = 6$) and TNBS-induced animals (PC: $n = 6$; MC: $n = 5$; DC: $n = 5$). * $p < 0.05$ vs. Ang II alone.

Since the results with L-NAME, atropine and phentolamine exhibited the same effects as TTX on Ang II-induced contraction (increased), we explored the possibility of the correspondent mediators (NO, muscarinic receptors and α-adrenoceptors, respectively) being dependent on prejunctional neuronal activation (evidenced by the effect of TTX). Co-administration of TTX and L-NAME did not alter the colonic contractile response to Ang II vs. TTX alone in the MC of control animals or in the PC and DC of TNBS-induced rats ($p > 0.05$; data not shown). Similarly, co-administration of TTX and atropine did not alter the colonic contractile response to Ang II vs. TTX alone in the MC of control animals or in the PC of TNBS-induced rats ($p > 0.05$; data not shown). The response to Ang II was

also not altered when the PC of control rats was incubated with TTX or with TTX plus phentolamine ($p > 0.05$; data not shown). However, co-administration of TTX and L-NAME and of TTX and atropine further increased the colonic contractile response to Ang II vs. TTX alone in the PC of control animals (Figure 8). Overall, these results suggest that in the PC of control rats, NO and muscarinic receptors participate in Ang II-induced contraction (decrease it) but not as a part of a mechanism involving neurons. Differently, the role for NO, muscarinic receptors and α-adrenoceptors in mediating Ang II-induced contraction in other colonic regions of control and TNBS-induced rats is dependent on the activation of enteric nerve terminals.

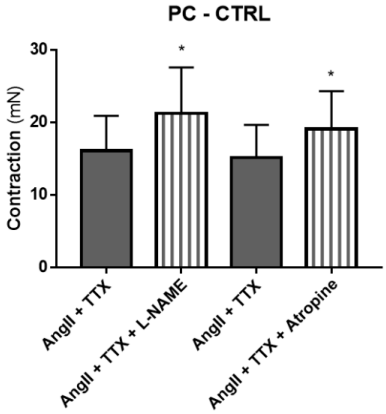

Figure 8. Contractile response to Ang II in the PC of control animals in the presence of TTX 1 μM (dark gray bars) compared to the presence of TTX and L-NAME 100 μM ($n = 6$) or atropine 1 μM ($n = 5$) (striped bars). * $p < 0.05$ vs. Ang II with TTX.

3. Discussion

Our study shows for the first time that TNBS-induced colitis is associated with decreased Ang II-mediated contraction along the entire rat colon. We also report evidence that Ang II-mediated contraction results from an intricate network between smooth muscle cells, nerve terminals, ICC and EGC, communicating through muscarinic receptors and NO release. Furthermore, we show that this complex mechanism is altered in rat TNBS-induced colitis (Figure 9).

IBD can affect scattered parts of the colon in Crohn's disease or continuously, with a distal-to-proximal pattern, in ulcerative colitis. Our study advances the state-of-the-art and pursues translational relevance in colitis because instead of studying the colon as a uniform target organ of IBD, we studied: (1) the PC—no macroscopic alterations, similar MPO activity but increased wet-to-dry ratio vs. controls; (2) the MC—evident macroscopic alterations and increased MPO activity and wet-to-dry ratio; and (3) the DC—increased MPO activity and wet-to-dry ratio than in controls but more marked MaS than the MC. With this approach, our study is the first to report a generalized decrease in the response to Ang II along the colon of rats with TNBS-induced colitis when compared to control rats. However, because we looked for the absolute and relative forces of contraction induced by Ang II, we could also report interesting differences in this decreased response to Ang II. Indeed, we report that minimum inflammatory alterations (PC) equally affect Ang II and ACh contractile function, while evident inflammatory damage (MC) further decreases the relative contractile effect of exogenous Ang II over that of ACh. However, marked inflammatory damage (DC) seems to favor the contractile response of Ang II over that of ACh, which might represent a compensatory response to the loss of function caused by extensive inflammatory injury (Figure 2). Inflammatory damage can nonspecifically affect the contractile capacity of the colon by decreasing the ability of the smooth muscle to contract, either by direct lesion of smooth muscle cells or by steric disturbance of the contractile mechanics across the colonic wall [32]. However, it can also disturb the interplay between the cells/receptors/mediators operating in the colon wall [32].

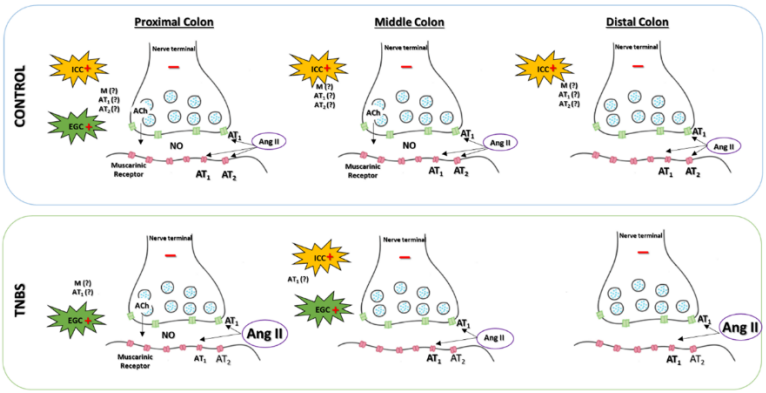

Figure 9. Schematic representation of the effect of Ang II in colonic reactivity along the colon of control (**top panel**) and TNBS-induced rats (**bottom panel**). Ang II activates AT_1 receptors, contracting all colonic regions of control and TNBS-induced rats. Ang II also activates AT_2 receptors along the colon of control animals, promoting colonic relaxation, but this effect is not observed in TNBS-induced rats. Animals with TNBS-induced colitis show higher tissue concentration of endogenous Ang II and decreased expression of AT_1 receptors but similar expression of AT_2 receptors. The intricate, Ang II-mediated colonic contraction involves a neuronal inhibitory component and a non-neuronal excitatory component. The neuronal component involves activation of muscarinic receptors and production of NO. The non-neuronal component includes activation of EGC and/or ICC, dependent on the colonic region. EGC—enteric glial cells; ICC—interstitial cells of Cajal; M—muscarinic receptor; NO—nitric oxide; (+)—excitatory mechanism; (−)—inhibitory mechanism.

The lower reactivity to Ang II might be the result of an imbalance in function and/or expression of Ang II receptors [33]. Our functional results with AT_1 (candesartan) and AT_2 (PD123319) antagonists showed that along the colon of control rats, Ang II-mediated contraction resulted from activation of AT_1 receptors, with a regulatory relaxation mediated by the AT_2 receptor that is blunted in TNBS-induced rats (Figure 4). This would anticipate an increase in Ang II-induced contraction in TNBS-induced rats, which would be in clear contrast with our findings. However, we observed higher tissue levels of Ang II (Figure 3), also reported in IBD patients [34,35], and these could contribute to downregulation of AT_1 receptor mRNA expression (as we observed), even without altering the expression of AT_2 receptors (Figure 5). This could justify the lower reactivity to Ang II observed in TNBS-induced rats when compared to controls. Although a contractile role for the AT_1 receptors has already been described in the healthy colon [15,36] and in experimental colitis [17], Zizzo and co-workers reported decreased Ang-II mediated contraction (% carbachol, an ACh mimetic) in the distal colon of DNBS-induced rats, associated with increased expression of both AT_1 and AT_2 receptors [17]. DNBS-induced colitis is a chemically-induced model of colitis very similar to that of TNBS, and experiments were also carried out after 1 week [17]. However, we used TNBS-induced animals that presented mild or moderate colitis [37], but the animals used by Zizzo et al. might have had more severe colitis, considering the reported MaS and MPO activity levels [17]. Furthermore, we report that two colonic segments just 2.5 cm apart (which we named MC and DC) present different relative responses to Ang II, while the authors mentioned above did not specify the exact location of the colon portion used. It is also possible that the downregulation of AT_1 receptors observed in our study serves as a mechanism to control TNBS-induced inflammatory damage because knock-out mice for AT_1 receptors have been shown to develop less severe colitis than wild-type mice [38,39].

Our results with TTX, NNC 55-0396 and FC support the view that the contractile effect of Ang II results from direct postjunctional activation of Ang II receptors in smooth muscle cells and also from indirect modulation of prejunctional release of neurotransmitters from nerve terminals, ICC and EGC [40–46]. Further, we distinguished not only a neuronal inhibitory regulation but also a non-neuronal (ICC, EGC) excitatory regulation, which is

altered in TNBS-induced colitis. Results with TTX support the view of an indirect inhibitory neuronal mechanism triggered by exogenous Ang II in all three regions of the colon of both control and TNBS-induced animals (Figure 6A). The study of Zizzo and collaborators also described an inhibitory neuronal pathway activated by Ang II in control animals but reported that it was blunted in DNBS-induced rats [17]. Other studies indicate that Ang II activates an excitatory neuronal mechanism in the mouse colon [15] and human sigmoid colon [36]. Non-neural cells such as ICC and EGC are also a major component of enteric neuromodulation [47] and play a role in the complex network that ensures proper colonic contraction [20,25,26]. To our knowledge, our study is the first to explore the putative role of ICC and EGC in Ang II-mediated contraction of the rat colon. Our results with NNC 55-0396 showed that in the three colonic regions of control rats, ICC contributed positively to Ang II-induced contraction, whereas for TNBS-induced animals, this mechanism was observed only in the MC (Figure 6B). Several studies have shown that ICC are lost or damaged in many inflammatory disorders, being decreased in the colons of patients with UC (inflamed regions compared to non-inflamed regions) [29] and in the rat TNBS model of ileitis [46] and colitis [48]. A role for ICC in synthesizing NO has also been suggested in experimental colitis [49]. Concerning EGC, our results show that they contribute to Ang II-induced contraction in the PC of control rats and in the PC and MC of TNBS-induced animals (Figure 6C), suggesting that during inflammation, Ang II activates EGC. Intriguingly, we found no effect of FC in the DC of TNBS-induced animals, which was the region presenting the most severe inflammatory damage, possibly resulting in loss of EGC [29]. Of interest, in a TNBS-induced model of ileitis in rats, inflammation stimulated EGC proliferation that was responsible for the maintenance of ACh release from enteric neurons [50]. Generally, TNBS-induced inflammation disturbs this intricate enteric network by altering the regulatory effect of non-neuronal cells: The regulatory role of the ICC seems to be lost, while that of the EGC appears to be recruited to support Ang II-induced contraction in a proximal-to-distal direction. Interestingly, it is reported that inflammation activates EGC, and that this could be associated with either a pro-inflammatory state or an attempt to resolve inflammation [22,23].

ACh and NO are the main neurotransmitters of the ENS, mediating contraction [51] and relaxation [52,53], respectively. Surprisingly, experiments with atropine revealed that activation of muscarinic receptors counteracted Ang II-mediated contraction in the PC and MC of control rats and in the PC and DC of TNBS-induced rats (Figure 7B), which is in contrast with previously reported data [15]. In vascular tissues, a role for direct M_3 receptor-mediated relaxation of the smooth muscle has been described [54,55], but in the colon, activation of M_3 receptors by ACh mediates contraction [56]. Even considering that Ang II decreases the release of ACh from nerve terminals [57], the effect of atropine should be a decrease in Ang II-mediated contraction. As such, these results further strengthen the view of an indirect pathway mediating Ang II contraction. Interestingly, our results with L-NAME showed a similar pattern to those with atropine (Figure 7A). ACh stimulates the production and release of NO from nerve terminals in the proximal [58,59] and distal [58] colon, but not in the middle colon [58,59]. Furthermore, ACh and NO might also act as cotransmitters in some enteric nerves [60] and exert neuromodulatory actions between them [61–63]. Our double antagonist protocol showed that in the PC of control rats, the inhibitory pathway involving both muscarinic receptors and NO is not (or not only) occurring through prejunctional nerves (Figure 8). Differently, in the other colonic regions, that inhibitory pathway seems to be entirely dependent on nerve terminals. Alterations in nitrergic neurotransmission occur in acute experimental colitis [53,64]. Zizzo et al. have previously suggested an inhibitory role for NO in modulating Ang II-induced colonic contraction in the distal colon of DNBS-treated rats [17], and it was reported that NO release from neurons or enteric glia is stimulated by the activation of nicotinic receptors in animals with experimental colitis but by muscarinic receptors in controls [64]. In an *Escherichia coli* endotoxin-induced model of colitis, expression of iNOS was found to be increased in the

inflamed colonic tissue, and its inhibition led to an increase in the contractile response to ACh, showing once again an interaction between the two molecules [65].

In conclusion, our study showed that in rats with mild/moderate experimental colitis caused by TNBS, Ang II-induced contraction was decreased. This possibly resulted from downregulation of AT_1 receptors and alterations in the communication between the intricate network of neuronal and non-neuronal cells (as ICC and EGC) by NO and muscarinic receptors.

4. Materials and Methods

4.1. Animals

Male Wistar Han rats were raised and maintained in the rodent animal facility of ICBAS-UP and housed 2–3 per cage in Rat IVC cages with controlled ventilation and a stable temperature (20–24 °C) and relative humidity (40–70%) under a regular 12 h light/dark cycle. All animals had *ad libitum* access to a laboratory rodent diet and tap autoclaved water. Enrichment materials like nesting paper and play tunnels were provided.

4.2. Colitis Induction

TNBS-induced colitis is a well-established animal model of IBD, widely used because it is simple to implement and cost-effective [31]. This model was introduced by Morris et al. [66], but several variations of the original protocol have been introduced [31,37]. Briefly, to achieve TNBS-induced colitis, an ethanolic TNBS solution should be instilled in the rectum. In this model, the alcohol is essential for disrupting the intestinal epithelial cell barrier, enabling TNBS to penetrate the bowel wall and resulting in haptenization of intestinal proteins, which signalizes them as targets of the host immune system [67].

Male Wistar Han rats of 8 to 12 weeks of age (littermates) were randomly assigned to the control or TNBS group. Control animals were not subjected to any manipulation. On day −1, rats of the TNBS group were individually housed in clean cages and fasted overnight with ad libitum access to a 5% sucrose solution. Then, on day 0, they were anesthetized ad effectum with isoflurane (Isoflo®, Esteve, Barcelona, Spain) and a 21% ethanolic solution of TNBS (Sigma-Aldrich Inc, St. Louis, MO, USA) (20 mg/rat; 250 µL) was rectally instilled (adapted from Morris et al. [66], using a 7.6 cm ball-tipped catheter (ST1 75-0285, Harvard Apparatus)). Rats were maintained in a head-down position for 60 s to ensure TNBS distribution and no leakage. Analgesia was provided, with daily administration of paracetamol (Paracetamol®, Farmoz, PO, 500 mg/kg) in a honey-based solution from day 0 until day 7. Tramadol (Labesfal, 100 mg/2 mL, SC) was only administered when animals presented severe signs of discomfort. In addition, metoclopramide (Labesfal, 1 mg/kg, SC) was administered from day −1 (before induction) to day 2 of the protocol to assure intestinal motility. All animals were monitored daily throughout the protocol to ensure their well-being and to record individual body weight and the number of fecal pellets. No mortality was observed in this study.

4.3. Evaluation of Macroscopic and Microscopic Inflammatory Damage

On day 7 or 8, animals were sacrificed by decapitation (Small Decapitator, Harvard Apparatus). The abdomen was opened, and the general appearance of the colon and surrounding tissues was observed. Next, the colon was excised and gently cleaned of fecal content using Krebs–Henseleit solution ((in mM): 118 NaCl, 4.8 KCl, 2.5 $CaCl_2 \cdot 2H_2O$, 1.2 $NaH_2PO_4 \cdot H_2O$, 1.2 $MgSO_4 \cdot 7H_2O$, 25 $NaHCO_3$, 0.02 Na_2EDTA, 0.3 Ascorbic Acid and 11 glucose mono-hydrated). Following that, four intact, 1 cm-long segments were cut as previously reported [37] and used in subsequent in vitro functional experiments (see below): two segments from the proximal colon (PC), 1 cm distal to the cecum; one segment from the middle colon (MC), 4.5 cm above the anus; and another segment from the distal colon (DC), 2 cm above the anus. These 3 areas were chosen to represent an area without obvious lesions (PC), an area upstream of the visible area of injury (MC) and another area close to the obvious area of injury (DC).

Subsequently, the rest of the colon was opened longitudinally to allow macroscopic evaluation of mucosal TNBS damage and attribution of a macroscopic score (MaS), as previously described [37] (Table 2). MaS was calculated as the mean between partial MaS of MC and DC because PC was the region without obvious macroscopic alterations. MaS assessment allowed categorization of experimental colitis in mild, moderate or severe colitis [37]. Only animals that presented mild or moderate colitis were used in this study.

Table 2. Macroscopic score (MaS).

Macroscopic Score (MaS)		Adhesions to Adjacent Organs	Colon Thickness	Mucosal Edema/ Hyperemia	Mucosal Ulcers
Score	0	Absent	Normal	Absent	Absent
	1	Mild/focal	Mild	Mild	Single
	2	Moderate/zonal	Moderate	Moderate	At one site
	3	Severe/diffuse	Marked increased	Severe	At more sites

Microscopic inflammatory damage was evaluated in a 1.5 cm-long, full-thickness segment of the colon, as previously described [37]. For that, the segment was fixed in 10% buffered formalin, embedded in paraffin wax and cut in 3 µm, full-thickness sections that were stained with hematoxylin and eosin.

4.4. Myeloperoxidase Assay

Myeloperoxidase (MPO) activity, a marker for neutrophil inflammation, was assessed as reported by Dinis-Oliveira et al. [68]. Briefly, tissues from three colonic regions were excised, pat-dried with gauze, weighted and homogenized on ice in ice-cold, 50 mM phosphate buffer (1:4 *m/v*) with 0.1% (*v/v*) Triton X-100, pH 7.4, followed by centrifugation at 15.700 g for 10 min at 4 °C. Supernatants were stored at −80 °C until use. Protein quantification was performed according to Lowry et al [69], using bovine serum albumin as a standard.

For the MPO assay, supernatants were submitted to three cycles of snap freezing (−80 °C to room temperature). In 96-well plates, to each well was added 50 µL of supernatant and 50 µL of TMB (final concentration 7.5 mM) dissolved in dimethyl sulfoxide. Immediately before plate reading, 50 µL of H_2O_2 (final concentration 1.5 mM) dissolved in phosphate buffer ($Na_2HPO_4 \cdot 2H_2O$ 50 mM, pH 5.4) was added to each well. MPO activity was monitored by recording the absorbance increase at 655 nm at 37 °C for 3 min. One enzyme unit (U) was defined as the amount of enzyme that utilized 1 µmol of H_2O_2 per minute as described by Marquez and Dunford [70]. Results are expressed in U/g of protein ($\varepsilon = 3.9 \times 10^4\ M^{-1}\ cm^{-1}$).

4.5. Characterization of the Reactivity of Colonic Tissue to Angiotensin II

Intact, 1 cm-long segments of the colon of control and TNBS-induced rats were mounted in isolated organ baths containing Krebs–Henseleit solution, continuously aerated (95% O_2 + 5% CO_2) and kept at a constant temperature of 37 °C. Segments were subjected to an initial resting tension of 1 g and left to equilibrate for at least 20 min until spontaneous mechanical activity was observed. Next, all segments were challenged with acetylcholine (ACh, Sigma-Aldrich, St. Louis, MO, USA) 10 µM twice to confirm the viability and stabilization of the tissue. After that, increasing concentrations of Ang II (Sigma-Aldrich, St. Louis, MO, USA) were added to the baths in a non-cumulative manner (PC: 100 pM–30 nM; MC: 300 pM–100 nM; DC: 1 nM–300 nM) for approximately 2 min. To prevent tachyphylaxis, there were intervals of 1 h between the addition of different concentrations of Ang II.

Next, we performed a second set of experiments in order to characterize the receptor(s) mediating the contractile response to Ang II. For that, the contractile response to submaximal concentrations of Ang II (PC: 10 nM; MC: 30 nM; DC: 100 nM) was evaluated in the absence and presence of candesartan 10 nM (dissolved in physiological saline; a kind

gift from Dr. Fredrik Palm, Uppsala University, Sweden), an AT_1 receptor antagonist, or of PD123319 100 nM (Sigma-Aldrich, St. Louis, MO, USA), an AT_2 receptor antagonist. The antagonists were left in contact with the tissue for 20 min before the second response to Ang II was obtained.

After that, we wanted to evaluate which cells of the enteric nervous system were involved in the contractile response to Ang II. For that, we used the same protocol as described above but using drugs that inhibited the function of nerve terminals, EGC and ICC. Thus, the contractile response to the same concentrations of Ang II indicated above were tested in the absence and presence of tetrodotoxin (TTX) 1 μM (Sigma-Aldrich, St. Louis, MO, USA), fluorocitrate (FC) 100 μM (Sigma-Aldrich, St. Louis, MO, USA) or (1S,2S)-2-(2-(N-[(3-Benzimidazol-2-yl)propyl]-N-methylamino)ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphtyl cyclopropanecarboxylate dihydrochloride hydrate (NNC 55-0396) (PC: 10 μM; MC and DC: 30 μM) (Sigma-Aldrich, St. Louis, MO, USA). TTX was used as a neurotoxin because it inhibits Na^+ voltage-gated ion channels, thus inhibiting the firing of action potentials in neurons [71]. FC is a metabolic toxin that selectively inhibits the tricarboxylic acid cycle in glial cells by blocking the enzyme aconitase [72]. NNC 55-0396 was used as a pharmacological tool to inhibit the activity of the ICC because it selectively blocks T-type, high-voltage-activated Ca^{2+} channels and does not inhibit other ion channels [73].

Furthermore, we repeated the same protocol with different drugs to evaluate the mediators involved in colonic contraction in response to Ang II. We investigated the role of NO, a potent vasodilator, by decreasing its production with NG-nitro-L-arginine methyl ester (L-NAME, 100 μM, Sigma-Aldrich, St. Louis, MO, USA), an inhibitor of nitric oxide synthase (NOS). In addition, we tested some antagonists to investigate the role of the correspondent receptors, namely atropine 1 μM (Sigma-Aldrich, USA) for muscarinic receptors, caffeine 300 μM (Sigma-Aldrich, St. Louis, MO, USA) for adenosine receptors, suramine 100 μM (Sigma-Aldrich, St. Louis, MO, USA) for P2-purinoceptors, phentolamine 3 μM (Sigma-Aldrich, St. Louis, MO, USA) for α-adrenergic receptors and propranolol 1 μM (Sigma-Aldrich, St. Louis, MO, USA) for β-adrenergic receptors.

Finally, we performed a third set of experiments in order to evaluate which cell was responsible for the release of the enteric mediators. In this experimental protocol, colonic segments were first incubated with TTX 1 μM for 30 min, and the response to Ang II (PC: 10 nM; MC: 30 nM; DC: 100 nM) was recorded. Afterward, tissues were washed for 1 h, every 10 min, and then co-incubated for 30 min with TTX 1 μM plus L-NAME 100 μM, atropine 1 μM or phentolamine 3 μM, which were added to the organ bath 10 min after TTX 1 μM (20 min incubation time). After this double incubation, colonic segments were again challenged with Ang II (PC: 10 nM; MC: 30 nM; DC: 100 nM).

At the end of each experimental protocol, tissues were dried on filter paper and weighed at the end of the experimental protocol, left overnight at room temperature and then weighed again in order to quantify the wet-to-dry ratio.

4.6. Extraction and Quantification of Angiotensin II Levels in Colonic Samples from PC, MC and DC

Immediately after the euthanasia of some animals (control and TNBS-induced), colonic portions (PC, MC and DC from control and TNBS-induced rats) weighting about 150–250 mg were collected. Tissues were homogenized with 5 mL homogenization buffer (sodium phosphate 0.1 M, sucrose 0.34 M and NaCl 0.3 M) with a protease inhibitor cocktail (Roche Complete mini #11836153001—one pill dissolved in 1 mL of distilled water; 100 μL of this solution was added to the homogenization buffer). Next, homogenates were centrifuged at 10000 rpm for 10 min at 4 °C, and supernatants were immediately separated to a different vial and kept frozen at −80 °C until further analysis.

Ang II was extracted by solid phase extraction (SPE) (Discovery®DSC-Ph SPE Tube, Supelco, PA, USA). SPE columns were activated using solvents in the following order: methanol, tetrahydrofuran, hexane, methanol and ultrapure water. Next, samples were applied to each column and eluted with ultrapure water. The aqueous extract was kept

at −20 °C for re-extractions, if needed. Following that, ultrapure water followed by a solution of acetic acid 4% were passed through the columns and discharged. Finally, a mixture consisting of ethanol:acetic acid:water (90:4:6) (this mixture should be prepared on time from cold stock solutions) was used to elute Ang II. Samples were concentrated by evaporation with nitrogen and then lyophilized and kept frozen at −80 °C until needed.

Quantification of endogenous tissue Ang II levels in the samples from PC, MC and DC (control and TNBS-induced rats) was performed using a peptide enzyme immunoassay (EIA) (Peninsula Laboratories International, Inc., San Carlos, CA, USA), following manufacturers' instructions (Protocol II). Results are presented as pg of Ang II per g of colonic portion (pg/g).

4.7. Expression of Angiotensin II Type I and II Receptors in Colonic Samples from PC, MC and DC by qPCR

Prior to sampling for RNA, colonic portions (PC, MC and DC of control and TNBS-induced rats) were excised and collected in a tube containing RNAlater®and kept at −20 °C until analysis. Total RNA was then extracted from 29.17–194.00 mg colonic tissue using PurezolTM RNA Isolation Reagent (Biorad, Hercules, CA, USA), and reverse transcription was performed from 1 µg RNA using the Xpert cDNA Synthesis Kit (GRiSP Research Solutions, Porto, Portugal) according to the manufacturer's instructions. RNA quantification and purity were measured spectrophotometrically at 260/280 in a plate reader (BioTek Synergy) adapted for 2 µL volume readings (Take3 adapter, BioTek, Winooski, VT, USA). Real-time polymerase chain reactions (qPCRs) were carried out applying SsoAdvanced™ Universal SYBR®Green Supermix (BioRad, Hercules, CA, USA) and run on a Bio-Rad CFX 96 thermalcycler using rat-specific primer pairs for the receptors AT_1 and AT_2, shown in Table 3 [74]. Data was normalized to the expression of housekeeping genes Papbn, Hprt-1 and Hmbs, whose expression was altered by sex, using the delta-delta C_t ($\Delta\Delta C_t$) method [75]. Every biological replicate was run in three qPCR technical triplicates. The thermal cycling protocol was the following: polymerase activation, 30 s at 98 °C, then denaturation for 15 s at 95 °C, followed by annealing/extension and plate read, 30 s at 60 °C, for 40 cycles.

Table 3. qPCR conditions for AT_1 and AT_2 receptors' expression analysis.

Genes of Interest		
AT_1 receptor	**Forward**	5′-TCTGGATAAATCACACAACCCTC-3′
	Reverse	5′-GAGTTGGTCTCAGACACTATTCG-3′
AT_2 receptor	**Forward**	5′-CTGGCAAGCATCTTATGTAGTTC-3′
	Reverse	5′-ACAAGCATTCACACCTAAGTATTC-3′
Housekeeping Genes		
Papbn-1	**Forward**	5′-TATGGTGCGACAGCAGAAGA-3′
	Reverse	5′-TATGCAAACCCTTTGGGATG-3′
Hprt-1	**Forward**	5′-CCCAGCGTCGTGATTAGTGATG-3′
	Reverse	5′-TTCAGTCCTGTCCATAATCAGTCC-3′
Hmbs	**Forward**	5′-TCTAGATGGCTCAGATAGCATGCA-3′
	Reverse	5′-TGGACCATCTTCTTGCTGAACA-3′

4.8. Data Collection and Statistical Analysis

For each experiment on the response to Ang II in each colonic segment, a concentration–response curve for Ang II was generated by best fit nonlinear regression (variable slope) using GraphPad Prism 7 software (La Jolla, CA, USA), and the correspondent Emax (maximum contractile effect induced by Ang II) and EC_{50} (concentration of Ang II that caused 50% of the maximum response) were obtained. Average concentration–response curves were generated for the response to Ang II obtained in tissues from control and TNBS-induced rats.

All data are mean ± S.E.M.; when stated, n refers to the number of experimental animals. GraphPad Prism 7 software was used for statistical analysis. An unpaired Student's t test was used for comparisons between control and TNBS-induced rats in the following analyses: Emax and EC_{50} values, dry tissue weight and wet-to-dry, Ang II content of each colonic segment and AT_1 and AT_2 expression as well as MPO activity. To evaluate the effect of the antagonists in the response to a single concentration of Ang II, a paired Student's *t* test was used. Two-way ANOVA was used to look for interactions between two factors. Anywhere, a *p* value lower than 0.05 was considered statistically significant.

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Institutional Review Board Statement: All animal procedures were performed according to the Portuguese DL n° 113/2013 and European Guidelines for humane and responsible animal care (European Directive 2010/63). All protocols were approved by local (179/2017 ORBEA ICBAS-UP) and national (003511/2018 DGAV) animal welfare bodies and are in accordance with the ARRIVE guidelines for reporting experiments. Animals were housed at the ICBAS-UP rodent animal facility, which is approved by the national competent authority (024159/2017 DGAV).

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TRABALHO 5

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ACE and ACE2 catalytic activity in the fecal content along the gut

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Abstract

Background: Angiotensin-converting enzyme (ACE) and ACE2 are two major enzymes of the renin–angiotensin–aldosterone system (RAAS), which control the formation/degradation of angiotensin (Ang) II and Ang1-7, regulating their opposite effects. We aimed at evaluating the catalytic activity of ACE and ACE2 in the intestinal content and corresponding intestinal tissue along the gut of Wistar Han rats.

Methods: Portions of the ileum, cecum, proximal colon, and distal colon, and the corresponding intestinal content were collected from Wistar Han rats. Enzyme activity was evaluated by fluorometric assays using different substrates: Hippuryl-His-Leu for ACE-C-domain, Z-Phe-His-Leu for ACE-N-domain, and Mca-APK(Dnp) for ACE2. ACE and ACE2 concentration was assessed by ELISA. Ratios concerning concentrations and activities were calculated to evaluate the balance of the RAAS. Statistical analysis was performed using Friedman test followed by Dunn's multiple comparisons test or Wilcoxon matched-pairs test whenever needed.

Key Results: ACE and ACE2 are catalytically active in the intestinal content along the rat gut. The ACE N-domain shows higher activity than the C-domain both in the intestinal content and in the intestinal tissue. ACE and ACE2 are globally more active in the intestinal content than in the corresponding intestinal tissue. There was a distal-to-proximal prevalence of ACE2 over ACE in the intestinal tissue.

Conclusions & Inferences: This work is the first to report the presence of catalytically active ACE and ACE2 in the rat intestinal content, supporting future research on the regulatory role of the intestinal RAAS on gut function and a putative link to the microbiome.

KEYWORDS
angiotensin-converting enzyme (ACE), angiotensin-converting enzyme 2 (ACE2), intestine, renin–angiotensin–aldosterone system (RAAS), stools

1 | INTRODUCTION

The renin–angiotensin–aldosterone system (RAAS) is a multienzymatic cascade traditionally associated with blood pressure regulation and fluid homeostasis.[1,2] Angiotensin (Ang) II, its main effector peptide, is mostly generated by the cleavage of Ang I by angiotensin-converting enzyme (ACE), and promotes vasoconstriction, pro-inflammatory, and fibrotic effects (classic RAAS).[1–3] Also, AngII is metabolized by ACE2 into Ang1-7 that mediates the opposite effects (counter-regulatory RAAS). Further, AngI can also be converted to Ang1-9 by ACE2, and then to Ang1-7 by ACE.[1,2,4] ACE and ACE2 are membrane-bounded carboxypeptidases with a single transmembrane helix and an intracellular segment, but are also found in their soluble form, which results from shedding of their extracellular domain.[5–7] While ACE2 has only one catalytic domain,[8,9] the extracellular domain of ACE has two catalytic domains (N- and C-domains) with different specificity and affinity for different substrates: the C-domain predominantly hydrolyzes AngI into AngII, while the N-domain is responsible for the metabolism of Ang1-7 into Ang 1-5, as well as other bioactive peptides[10–12] (Figure 1). ACE and ACE2 are the major RAAS enzymes and ACE2/ACE balance is essential for determining RAAS activity since it reflects the activation of the classic and counter-regulatory arms and, subsequently, overall RAAS-mediated effects.

Besides its classical view as an endocrine circulating system, several RAAS have been found to exist locally in different organs and tissues,[13] with an independent regulation of the circulating RAAS.[2] For instance, RAAS enzymes and peptides have been found in the gastrointestinal (GI) tract,[13–16] where they play a fundamental role in GI physiology and pathophysiology. Intestinal AngII regulates glucose absorption, water, and electrolytes homeostasis or bicarbonate secretion.[15] Also, we and others reported that AngII is also responsible for regulating intestinal smooth muscle tone,[17–19] through a complex network of intestinal cells as neurons, interstitial cells of Cajal and enteric glia, thus regulating normal intestinal motility. RAAS enzymes can also show RAAS-independent effects associated with the intestinal function. ACE participates in peptide digestion[13] and ACE2 is crucial for the intestinal expression of the neutral amino acid transporter B^0AT1, thus regulating the nutritional environment of the gut and the composition of the microbiota.[20] In fact, an interaction between the intestinal RAAS and microbiota has been suggested.[21] This interaction is bidirectional: genetic deletion of the counter-regulatory Mas receptor alters gut microbiota[22] while GI microbiota reduces ACE2 intestinal expression[23–25] and a bacterial aspartate beta-decarboxylase is fundamental for the endogenous synthesis of alamandine,[26] the newest RAAS peptide.

Although there is a GI RAAS and the referred evidence of an interaction with the gut microbiota, information on the presence of a functional RAAS in the stools or intestinal content remains scarce. In fact, to our knowledge, there are only two scientific papers that assessed the presence/activity of ACE in the human ileal fluid[27] and human stools of healthy individuals, this being altered in patients with celiac disease.[28] No one has looked for fecal ACE2. So, the aim of this work was to investigate the concentration and catalytic activity of ACE and ACE2 throughout the intestinal content and excreted fecal pellets of Wistar rats, and to compare the results on the intestinal content with that of the corresponding intestinal tissue.

Key Points

- ACE and ACE2 activity is found in the rat intestinal content along the GI tract.
- ACE is more prevalent in the intestinal tissue than in the intestinal content.
- ACE2 is more prevalent in the intestinal content than in tissue (except the ileum).
- ACE and ACE2 are more active in intestinal content than in intestinal tissue.

2 | MATERIALS AND METHODS

2.1 | Animals and sample collection

This project was approved by the local (179/2017-ORBEA-ICBAS-UP) and national (003511/2018-DGAV) competent authorities, and was reported in accordance with the ARRIVE guidelines[29] and the EU Directive 2010/63/EU for animal experiments. Eight male specific-pathogen free Wistar Han rats, 12 weeks old, weighting 344.0 ± 49.1 g, were individually caged in conventional cages for 24 h before the day of sample collection. Animals had *ad libitum* access to autoclaved tap water and standard laboratory rodent diet (4RF21, Mucedola S.R.L.). Enrichment material such as nesting material and paper tunnels was provided to all cages. For reduction purposes (3Rs), animals were shared with ongoing study that only included male rats.

On the day of the experiment, animals were weighted and then sacrificed by decapitation. Subsequently, the abdomen was opened and the intestine was collected from the rectum until the stomach. Then, samples from distal and proximal colon (DC and PC, respectively), cecum, and terminal ileum were collected. These portions were opened through the mesenteric border, allowing the collection of the intestinal content directly from the corresponding intestinal tissue portion, to avoid contamination. Fecal pellets from the cages were also collected. Other intestinal portions, as well as other organs, were used for other ongoing projects (3Rs—reuse).

Each intestinal portion was minced to pieces, randomized within assays, and then homogenized. Intestinal content and cage fecal pellets (CFPs) were also homogenized.

For enzyme activity assay, intestinal tissue and content, and CFPs were homogenized (200 mg tissue/feces to 1 mL of buffer) in a buffer containing: 100 mM sodium borohydride buffer, pH 7.2, 340 mM sucrose, 300 mM NaCl, and 1 mM phenylmethylsulphonyl fluoride (PMSF) inhibitor. PMSF was prepared as a concentrated

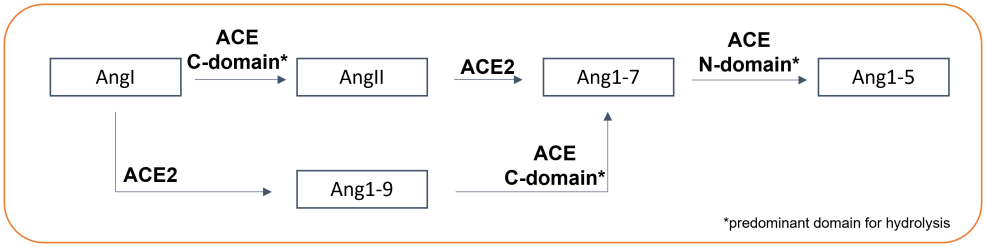

FIGURE 1 Schematic representation of simplified overview of the renin–angiotensin-aldosterone system. ACE, angiotensin-converting enzyme; Ang, angiotensin.

solution of 200 mM, and then added to the homogenization tubes at the time of homogenate preparation to get the final concentration of 1 mM. Buffer preparation as well as sample homogenization were performed on ice. Samples were then centrifuged at 3000 rpm, at 4°C, for 15 min. Supernatants were collected and stored at −20°C (tissue) and −80°C (feces) until assayed.

For the enzyme concentration assay, intestinal tissue was homogenized in PBS (100 mg of tissue to 1 mL of PBS) and then centrifuged at 3000 rpm for 20 min. Supernatant was collected and stored at −20°C until assayed.

2.2 | ACE and ACE2 concentration

ACE and ACE2 concentrations in the homogenate supernatants of intestinal tissue were quantified using ELISA commercial kits (cat. no. abx255667, Abbexa®, for ACE; cat. no. MBS014209, MyBioSource, for ACE2) following the manufacturer's instructions, while total proteins were quantified according to Lowry et al.[30] using bovine serum albumin as a standard. Results were expressed as mg of enzyme/mg of total proteins.

2.3 | ACE and ACE2 activity

ACE activity was assessed as previously described.[31] Hippuryl-His-Leu (h-HL) and Z-Phe-His-Leu (Z-FHL) were used as substrates that characterize the activity of the C-domain and the N-domain, respectively: the C-domain hydrolyzes h-HL and AngI at a higher rate than the N-domain, and the N-domain hydrolyzes Z-FHL at a higher rate compared to the C-domain.

Briefly, 10 µL of the intestinal tissue or intestinal content homogenate was incubated for 10 min at 37°C with 200 µL of assay solution: 1 mM Z-FHL or 5 mM h-HL in 100 mM potassium phosphate buffer, pH 8.3, containing 300 mM NaCl and 0.1 mM $ZnSO_4$. Then, after addition of 1.5 mL of 0.28 M NaOH to stop the enzymatic reaction, samples were incubated with 100 µL of *o*-phthaldialdehyde (20 mg/10 mL in methanol) for 10 min at room temperature, to allow binding of *o*-phthaldialdehyde to the newly formed peptide HL, resulting in a fluorescent product. The reaction was then stopped by the addition of 200 µL of 3 N HCl, followed by centrifugation at 3000 rpm for 5 min at 4°C. The amount of hydrolysis product HL was measured fluorometrically ($\lambda_{excitation}$ = 360 nm; $\lambda_{emission}$ = 465 nm) using SpectraMax Gemini EM microplate reader (Molecular Devices). In the intestinal tissue, ACE activity was presented as global enzyme activity normalized by the concentration of total proteins (nmol/min/mg of total proteins) or normalized by the concentration ACE, to get the correspondent specific activity of the enzyme (nmol/min/ng of ACE). In the intestinal content, ACE activity was presented as global enzyme activity normalized by the concentration of total proteins (nmol/min/mg of total proteins).

ACE2 activity was performed by a fluorometric kinetic assay using 20 µM Mca-APK(Dnp) as a substrate (cat. no. BML-P163-0001, Enzo Life Sciences, Inc.).[32] In brief, buffer (complete mini EDTA-free [1 pill for 10 mL of buffer], 75 mM Tris, 1 M NaCl, 0.5 mM $ZnCl_2$, 10 µM captopril, pH 6.5, all from Merck®) and sample homogenates (10 or 5 µL for intestinal tissue or content, respectively) were preincubated for 5 min at 37°C in the presence or absence of the selective ACE2 inhibitor DX600 (Abcam plc—10 or 50 µM for intestinal tissue or content, respectively). The substrate was added and fluorescence was measured for 120 min every 2 min ($\lambda_{excitation}$ = 320 nm; $\lambda_{emission}$ = 420 nm) using a SpectraMax Gemini EM microplate reader (Molecular Devices). Arbitrary units were registered, calculations were done based on a fluorescence standard curve using OmniMMP® fluorogenic control (Cat. no. BML-P127-0001, Enzo Life Sciences, Inc.), and the time point 0 was used as the internal blank. Results are presented as global enzyme activity normalized by the concentration of total proteins (nmol/min/mg of total proteins) for intestinal tissue and intestinal content, or normalized by the concentration ACE2, to get the corresponding specific activity of the enzyme (nmol/min/ng of ACE2) in the intestinal tissue.

To assess the relative contribution of the concentration and activity of ACE2 and ACE in each sample, we calculated and analyzed several ratios: ACE N-domain/C-domain activity ratio; ACE2/ACE N-domain activity ratio; and ACE2/ACE C-domain activity ratio; ACE2/ACE concentration ratio.

2.4 | Statistical analysis

Data analysis was performed using GraphPad Prism (Graphpad Software). Data were tested for normality using the Shapiro–Wilk test (small sample size) and the evaluated parameters were found to have a non-normal distribution. Hence, we used a paired experimental

design and the Friedman test followed by Dunn's multiple comparisons test to compare parameters between the intestinal samples of different intestinal regions. Moreover, we used Wilcoxon matched-pairs test to compare each parameter in each intestinal region between the value of the intestinal tissue and that of the corresponding intestinal fecal content. Results are presented as median [interquartile range]. $p < 0.05$ was considered statistically significant.

Sample size calculation was based on the primary experimental outcome assessed on the original project (003511/2018-DGAV). Only control animals were used in this study and no criteria were set to include or exclude the animals. The experimental unit was considered the individual animal.

3 | RESULTS

3.1 | ACE2 and ACE are present and active along the rat gut

ACE2 concentration was similar across the intestinal regions studied (Figure 2A) but ACE concentration was higher in the DC than in the ileum and cecum (Figure 2B). In line with this, the ACE2/ACE concentration ratio was lower in the DC than in the ileum and cecum (Figure 2C); this ratio was lower than 1 in the DC, around 1 in the PC, but higher than 1 in the cecum and ileum (Figure 2C), showing that in the ileum ACE2 concentration prevails over ACE but this is attenuated along the gut so that in the DC ACE concentration prevails over ACE2.

ACE2 activity (Figure 3A) and ACE N-domain activity (Figure 3E) were similar among the intestinal regions studied, and the same was observed for ACE2 and ACE N-domain specific activities (Figure 3B,F). As for the ACE C-domain activity, the ileum showed increased global enzyme activity than the cecum and increased specific activity than the DC (Figure 3C,D).

The ratio between the activities of the N- and C-domains of ACE was higher than 1 and similar between all intestinal tissue samples (Figure 4A), suggesting that the intestinal tissue ACE activity occurs more at the N-domain than at the C-domain. Also, the ACE2/ACE C-domain activities ratio was over 1 in all samples but similar among them, except for an increase in cecum over DC that was not observed when specific activities were taken (Figure 4B,C). Moreover, there was no difference in the ACE2/ACE N-domain activities ratios between intestinal regions (Figure 4D,E), although these ratios were always lower than 1 for all regions except the cecum, where they were higher than 1 (Figure 4D) or just around 1 (Figure 4E). These results suggest that overall, in the intestinal tissue ACE N-domain is more active than ACE2 which is more active than the ACE C-domain.

3.2 | ACE2 and ACE are active in the rat intestinal content along the gut

ACE2 activity was similar among the intestinal content of the different regions studied, or the CFPs (Figure 5A). Regarding ACE activity, the catalytic activity in the ileal content was higher than that of DC for both catalytic domains (Figure 5B,C), and also higher than that of CFPs for the N-domain (Figure 5C).

The ratio between the activity of the ACE N- and C-domains was higher than 1 in all fecal samples and did not differ along the intestine (Figure 6A), again pointing to a higher activity of the N- over the C-domain of ACE. The ratios between the activity of ACE2 and that of either the N- or C-domains of ACE did not statistically differ between fecal samples (Figure 6B,C). However, the ACE2/ACE C-domain activity ratio was higher than 1 in all fecal samples except the ileal content (Figure 6B), and the ACE2/ACE N-domain activity ratio was higher than 1 only in the DC and CFP (Figure 6D). These results suggest that in the intestinal content (similar to that observed in the intestinal tissue), ACE N-domain is more active than ACE2 which is more active than the ACE C-domain.

3.3 | ACE2 and ACE activities are different between the intestinal content and anatomically corresponding intestinal tissue

ACE2 activity was higher in the intestinal content than the anatomically corresponding intestinal tissue in all intestinal regions, except

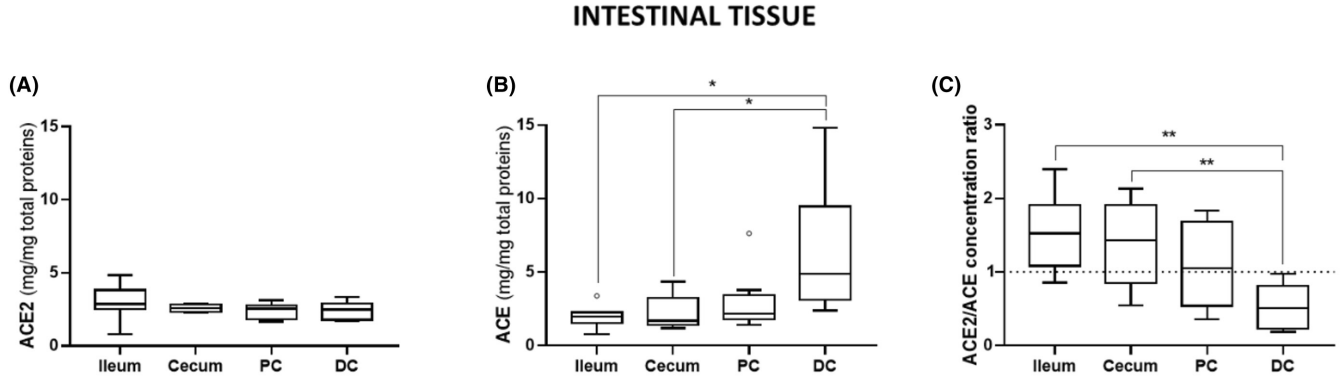

FIGURE 2 (A) Angiotensin-converting enzyme 2 (ACE2) concentration (mg/mg of total proteins), (B) ACE concentration (mg/mg of total proteins), and (C) ACE2/ACE concentration ratio along the gut ($n = 8$). DC, distal colon; PC, proximal colon. *$p < 0.05$, **$p < 0.01$.

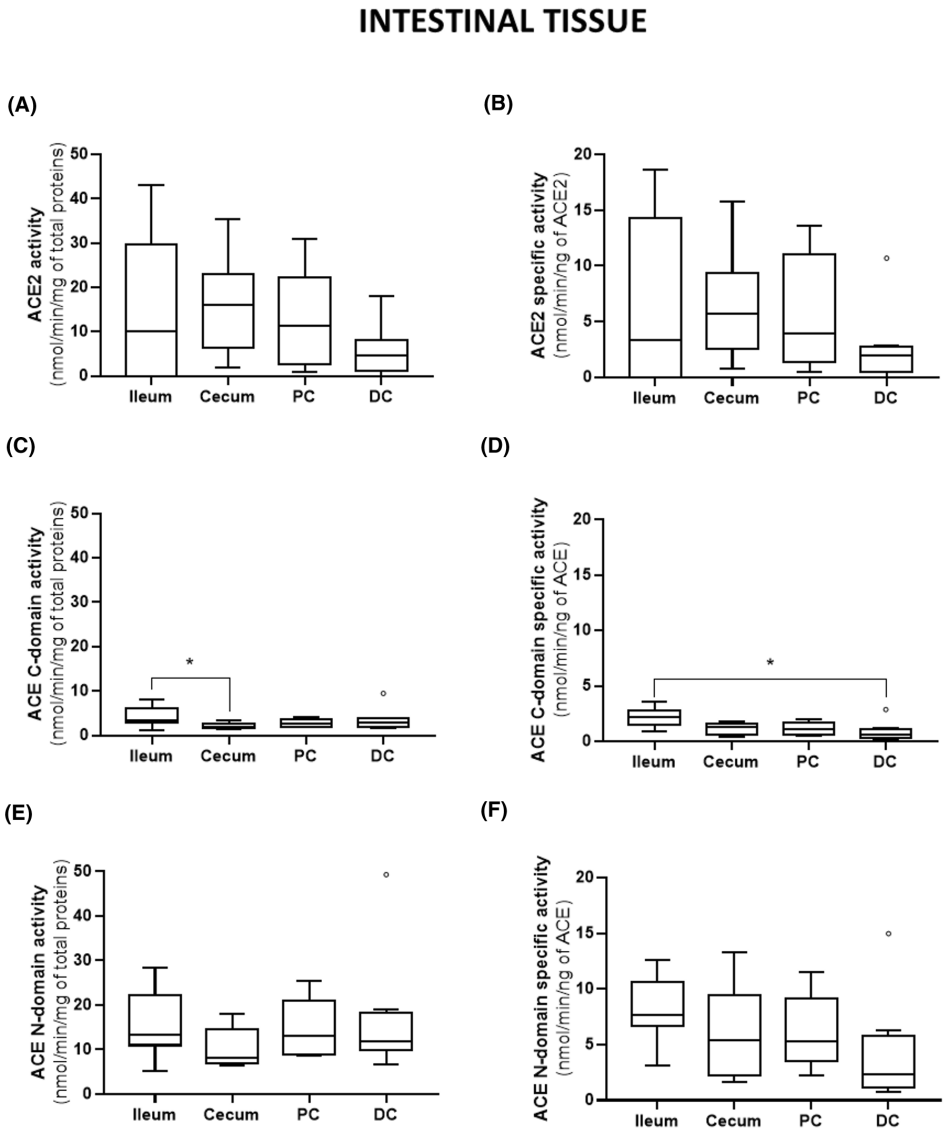

FIGURE 3 Activity (nmol/min/mg of total proteins) (A, C, E) and specific activity (nmol/min/ng of angiotensin-converting enzyme 2 (ACE2) or ACE) (B, D, F) of ACE2 (A, B) ACE C-domain (C, D), and ACE N-domain (E, F), along the gut ($n = 8$). DC, distal colon; PC, proximal colon. $^{**}p < 0.01$.

in the PC (Table 1). Regarding ACE activity, both C-domain and N-domain activities were higher in the intestinal content than the corresponding intestinal segment for all intestinal regions (Table 1).

Regarding activity ratios, no differences were found in the ACE-N-domain/C-domain activity ratio, neither in ACE2/ACE-C-domain or ACE2/ACE-N-domain activity ratios between intestinal content and corresponding intestinal tissue (Table 2).

4 | DISCUSSION

Our study has some groundbreaking findings that will enhance knowledge on the RAAS. First, both ACE and ACE2 are catalytically active along the rat intestinal content and CFPs. Second, ACE and ACE2 activities are higher in the intestinal content than the corresponding intestinal tissue. Finally, the balance between classic and counter-regulatory arms of the RAAS seems to be similar in the intestinal content and the intestinal tissue.

The mapping of the activity of both ACE and ACE2 along the rat intestinal content is groundbreaking. To our knowledge, the presence of active ACE has only been referred in two reports published in the 1990s. Deddish *et al*. described the presence of an active N-domain ACE isoform in the ileal fluid of patients that underwent surgical colectomy[27] and Letizia *et al*. reported a non-differentiated ACE activity in human stools.[28] However, neither of these research groups explored this interesting finding, which we have now started to characterize. Innovatively, our study shows that while ACE2 is equally active along the content of the intestinal tract, ACE activity (both N- and C- domains) is higher in the ileal content than in more distal regions or CFPs. Although further studies are needed, these results prompt us to suggest the existence of a local fecal RAAS with both classical and counter-regulatory arms actively present.

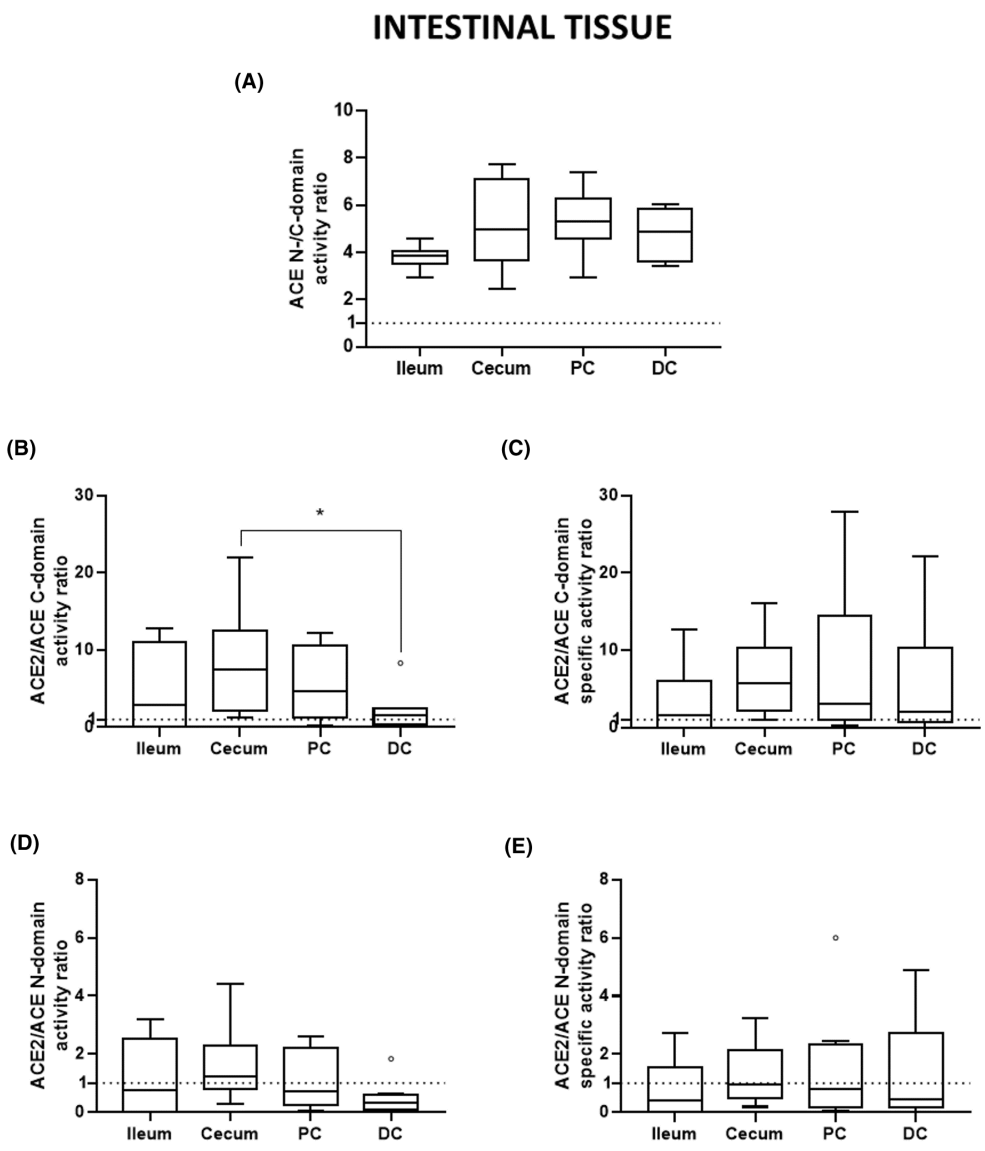

FIGURE 4 Activity (A, B, D) and specific activity (C, E) ratios of (A) angiotensin-converting enzyme (ACE) N-/C-domain, (B, C) ACE2/ACE C-domain, and (D, E) ACE2/ACE N-domain along the gut ($n = 8$). DC, distal colon; PC, proximal colon. $^{*}p < 0.05$.

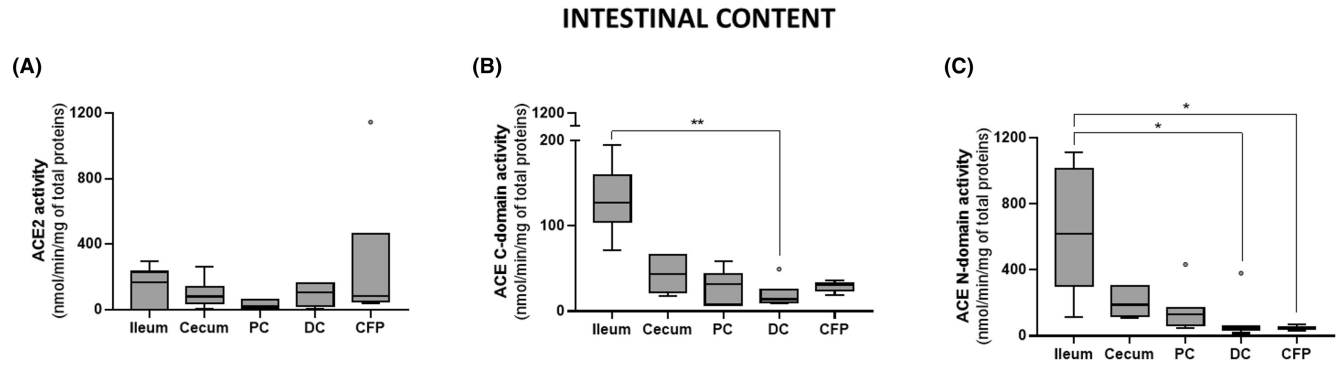

FIGURE 5 Activity (nmol/min/mg of total proteins) of (A) angiotensin-converting enzyme 2 (ACE2), (B) ACE C-domain, and (C) ACE N-domain in the intestinal content along the gut ($n = 8$). CFP, cage fecal pellets; DC, distal colon; PC, proximal colon. $^{*}p < 0.05$, $^{**}p < 0.01$.

Due to the close connection between the intestinal content and tissue, we also mapped these enzymes in the intestinal tissue and reported that both enzymes are present and active along the rat gut. The view of a local intestinal RAAS is not new,[13,15] although studies do not simultaneously characterize different intestinal regions, neither include together data on enzyme concentration and activity.

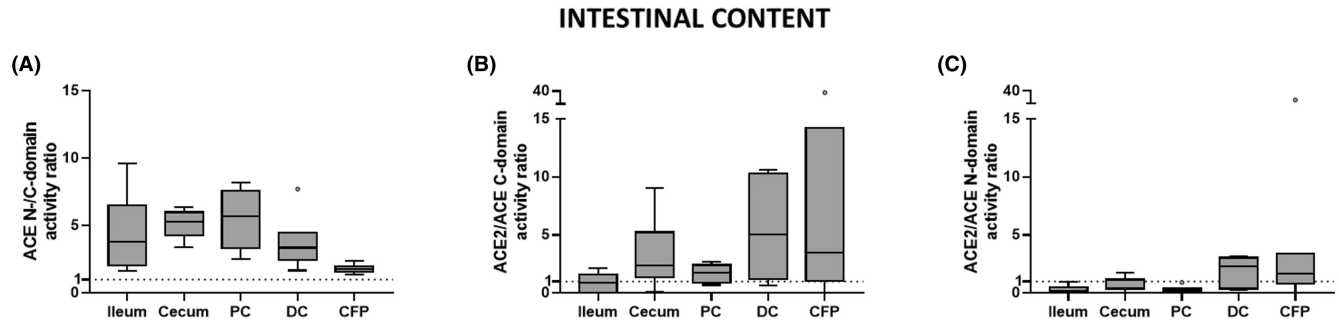

FIGURE 6 Activity ratios of (A) angiotensin-converting enzyme (ACE) N-/C-domain, (B) ACE2/ACE C-domain, and (C) ACE2/ACE N-domain in the intestinal content along the gut ($n = 8$). CFP, cage fecal pellets; DC, distal colon; PC, proximal colon.

TABLE 1 Angiotensin-converting enzyme 2 (ACE2), ACE C-domain, and ACE-N-domain activity in the intestinal content and the corresponding intestinal tissue ($n = 8$).

	Intestinal content	Intestinal tissue	*p*-Value
ACE2 activity (nmol/min/mg of total proteins			
DC	106 [23.3–160]	3.88 [0.412–6.76]	**0.0156**
PC	21.5 [13.3–59.0]	7.63 [2.34–22.2]	0.2969
Cecum	80.9 [17.1–181]	16.0 [6.33–23.0]	**0.0234**
Ileum	155 [21.3–200]	10.0 [0.00–29.7]	**0.0391**
ACE C-domain activity (nmol/min/mg of total proteins)			
DC	14.1 [10.1–25.1]	2.79 [1.83–3.60]	**0.0313**
PC	34.3 [7.91–38.8]	2.71 [2.01–3.95]	**0.0156**
Cecum	30.8 [19.0–63.1]	1.99 [1.52–2.59]	**0.0078**
Ileum	127 [82.4–182]	3.41 [2.99–6.01]	**0.0078**
ACE N-domain activity (nmol/min/mg of total proteins)			
DC	48.9 [36.2–70.9]	11.8 [10.0–18.4]	**0.0078**
PC	127 [65.0–171]	13.0 [9.18–22.6]	**0.0156**
Cecum	190 [118–282]	8.14 [6.96–14.6]	**0.0078**
Ileum	526 [269–962]	13.4 [11.0–22.2]	**0.0078**

Note: $p < 0.05$ was considered statistically significant (bold).
Abbreviations: DC, distal colon; PC, proximal colon.

ACE2 was evenly distributed and active from the terminal ileum to the DC. These data are in accordance with data from other studies reporting that ACE2 is expressed throughout the small and large intestine,[9,33–37] although some studies have reported that ACE2 expression is higher in the ileum than in the colon.[34–37] Intestinal ACE2 actively removes C-terminal single neutral amino acid from nutrient peptides, which are then uptaken by the neutral amino acid transporter B^0AT1.[38,39] Interestingly, it has been reported that ACE2 expression is required for the expression, proper trafficking, and functioning of B^0AT1,[39] which is abundantly expressed in the ileum.[40] Accordingly, in ACE2 knockout mice, tryptophan uptake was impaired, leading to compromised antimicrobial peptide formation and altered gut microbiota composition.[20,41] In our study, ACE concentration was higher in the DC than in the terminal ileum and cecum, although its C-domain was more catalytically active in the ileum than in the DC (with no difference for the N-domain). ACE was also reported to be expressed in the small and large intestine.[13,15,16] But, Erickson *et al.* have reported that within the small intestine, ACE mRNA and protein, as well as enzymatic activity, are higher in the proximal to middle portions, decreasing toward the distal end,[42] which was the only region of the small intestine that we studied. Despite this observation, we are, to our knowledge, the first group to report differences in ACE concentration between small and large intestinal portions. ACE may also have a role in digesting dietary proteins. Yoshioka *et al.* described that ACE, present in the intestinal brush-border membrane, can cleave C-terminal residues from peptides at the same hydrolysis rate than other intestinal brush-border membrane peptidases like aminopeptidase IV and aminopeptidase N.[13,43] Interestingly, ACE and ACE2 seem to be more abundant in the intestine than in other tissues and fluids.[35,44]

In order to understand the relation between the fecal RAAS and the intestinal RAAS, we compared ACE and ACE2 activities between the intestinal content and the anatomically corresponding intestinal tissue. Curiously, we observed that both ACE (N- and C-domains) and ACE2 activities are higher or similar (never lower) in the intestinal content than in the corresponding intestinal tissue. The presence of catalytically active ACE and ACE2 in the intestinal content could simply result from enzyme shedding from the intestinal epithelial membrane. However, if this should be the only origin of enzyme activity in the intestinal content, and considering that our study used healthy rats, one would expect that the enzyme activity in the intestinal content would be similar or lower than that of the intestinal membrane. Hence, we speculate that the intestinal microbiota can be a source of enzyme activity in the intestinal content. New studies are ongoing in order to test this hypothesis but, at present, the possibility is already thrilling since it would unravel the existence of a microbiota RAAS. It has been suggested that the RAAS and the microbiota interact.[21] However, studies underlying this connection have focused on intestinal microbiota and local tissue intestinal RAAS. Indeed, GI microbiota reduces intestinal ACE2 mRNA expression,[23–25] although it is not clear whether the variability in the composition of the microbiota determines the degree of ACE2 expression.[23,25] Hashimoto *et al.* have shown that germ-free mice receiving microbiota from ACE2 knockout mice presented a more severe colitis, highlighting the important relationship between GI microbiota, ACE2, and GI homeostasis.[20] From another perspective, knockout of the Mas receptor (main receptor of the counter-regulatory arm of the RAAS) alters

	Intestinal content	Intestinal tissue	*p*-Value
ACE N-domain/C-domain activity ratio			
DC	3.37 [2.43–4.49]	4.55 [3.57–6.03]	0.4375
PC	4.97 [3.63–7.39]	5.51 [4.56–6.45]	>0.9999
Cecum	5.28 [4.18–6.23]	4.97 [3.65–7.10]	0.7422
Ileum	4.99 [2.20–6.65]	3.87 [3.52–4.05]	0.5489
ACE2/ACE C-domain activity ratio			
DC	1.42 [0.307–2.99]	0.311 [0.0743–0.596]	0.1563
PC	0.284 [0.136–0.439]	0.729 [0.240–2.22]	0.0781
Cecum	0.386 [0.0995–1.08]	1.22 [0.804–2.27]	0.0781
Ileum	0.215 [0.0193–0.555]	0.759 [0.00–2.53]	0.3125
ACE2/ACE N-domain activity ratio			
DC	1.42 [0.307–2.99]	0.311 [0.0743–0.596]	0.1094
PC	0.284 [0.136–0.439]	0.586 [0.235–1.719]	0.1563
Cecum	0.386 [0.0995–1.08]	1.22 [0.804–2.29]	0.0781
Ileum	0.215 [0.0193–0.555]	0.769 [0.00–2.53]	0.3125

Note: $p < 0.05$ was considered statistically significant.
Abbreviations: DC, distal colon; PC, proximal colon.

TABLE 2 Ratios between angiotensin-converting enzyme (ACE) N-domain/C-domain activity, ACE2/ACE C-domain activity, and ACE2/ACE-N-domain activity in the intestinal content and the corresponding intestinal tissue ($n = 8$).

mouse intestinal microbiota.[22] Hence, we hypothesize that the differences found in our study might reflect the different composition of the microbiota between the intestinal regions.[45]

The balance of the RAAS activity relies considerably on the balance between the activities of its classic and counter-regulatory arms (Figure 1), which in our study are represented by the activities of ACE and ACE2, respectively. To get a general picture of the RAAS balance within our experimental setup, we also evaluated several enzyme ratios. Interestingly, although there seems to be a higher activity of each enzyme domain in the intestinal content than in the corresponding intestinal tissue, RAAS balance is preserved in the intestinal content when compared with that of the intestinal tissue, suggesting an equilibrium between intestinal and fecal RAAS. Globally, our results show that both in the rat intestinal content and in the tissue ACE N-domain is more active than ACE2 which is more active than the ACE C-domain. There is: (1) higher activity of the ACE N-domain over the C-domain, (2) higher activity of ACE2 over that of the ACE C-domain, but (3) lower activity of ACE2 over that of the ACE N-domain. ACE C-domain activity mainly determines the formation of AngII[11,12] (Figure 1), which is catabolized into Ang1-7 by ACE2[1,2,4] (Figure 1), and Ang1-7 is hydrolyzed into inactive peptides by the ACE N-domain[11,12,46,47] (Figure 1). If we reason on this RAAS picture and our ratio data, the higher ACE2/ACE C-domain activity ratio possibly results in more Ang1-7 being formed when compared to AngII. This would also explain the higher ACE N-/C-domain activity, which would contribute to regulate the increased formation of Ang1-7 by increasing its hydrolysis and decreasing the formation of AngII, which would promptly generate more Ang1-7 through the high ACE2 activity. Finally, the lower ACE2/ACE N-domain activity ratio observed in the more proximal intestinal regions would also contribute to regulate Ang1-7 concentration by increasing its hydrolysis and decreasing its formation. Although these interpretations are speculative, our study clearly shows a dynamic balance between ACE and ACE2 not only in the intestinal tissue but also, and innovatively, in the intestinal content.

Current knowledge of the RAAS prompts us to speculate on the pathophysiological relevance of our data. The imbalance between the abundance and/or activity of gut ACE and ACE2 may have an impact on: (1) smooth muscle tone and electrolyte homeostasis, (2) intestinal inflammation, (3) gut dysbiosis, (4) dysregulation of tryptophan metabolism, (5) dysregulation of metabolic pathways (e.g., glucose absorption), and (6) susceptibility to intestinal infections and associated intestinal manifestations.[15,21,48,49]

In conclusion, we report for the first time the presence of enzymatically active ACE and ACE2 along the intestinal content of the rat, with some regional differences and independence from the corresponding tissue. This work sheds new light on the RAAS, with the hint of the existence of a fecal RAAS. Quantification of other RAAS components, namely AngII and Ang1-7, in future studies will refine the present data. Our data open a totally unexplored line of research that might be of translational relevance in the field of fecal biomarkers of intestinal diseases, and others associated with the microbiota, as well as for the use of genetically modified probiotics (that would secrete ACE inhibitors or Ang1-7) in the treatment of inflammatory GI or cardiovascular diseases.

AUTHOR CONTRIBUTIONS

Mariana Ferreira-Duarte designed and performed the research, analyzed the data and wrote the manuscript. Lilian Caroline Gonçalves Oliveira, Clara Quintas, Marisa Esteves-Monteiro, Margarida Duarte-Araújo, and Teresa Sousa helped with parts of the research protocols and analysis of the correspondent data, and revised the final manuscript. Dulce Elena Casarini and Manuela Morato supervised the protocols, analyzed the data, and revised the final manuscript. Manuela Morato was also responsible for gathering the financial support of the research.

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CONFLICT OF INTEREST STATEMENT

The authors have no competing interests.

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